University of Alberta

Molecular and Hormonal Regulation of 5'AMP-Activated Protein Kinase in the Heart

by

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This thesis is dedicated to my wonderful
family and friends.

Jesus, Rosita,
Jenny,
David,
Eva,
&
Jukie

Thank-you for all of the smiles!

ABSTRACT

Alterations in myocardial metabolism are important determinants of functional recovery in the post-ischemic heart. 5'AMP-activated protein kinase (AMPK) is a pivotal regulator of key metabolic enzymes in the heart. While the mechanisms underlying the AMPK-dependent regulation of metabolism in the heart have been defined, the regulation of myocardial AMPK remains poorly understood. Thus, we conducted an in-depth characterization of the molecular and hormonal mechanisms that regulate AMPK in the heart.

In this thesis, stimulation of AMPK by ischemia is shown for the first time to be mediated by an AMPK kinase (AMPKK). Furthermore, a recently identified AMPKK, LKB1, is not activated by ischemia, supporting the existence of an LKB1-independent, ischemia-stimulated AMPKK. In efforts to identify an AMPKK that is distinct from LKB1, two rat heart AMPKKs were purified, neither of which co-purified with LKB1. Thus, LKB1 is but one of at least three AMPKKs expressed in mammalian cells.

In additional studies, the role of insulin-receptor signaling in the AMPKK-mediated regulation of AMPK was assessed. Since insulin inhibits AMPK activity and phosphorylation in aerobic and ischemic hearts, we investigated whether insulin also represses the activity of AMPKK and thus AMPK.

However, insulin did not alter the activity of AMPKK in either aerobic or ischemic hearts perfused with clinically relevant levels of fatty acids.

Recently, AMPK was identified as an effector of signaling from the adiponectin/globular domain of adiponectin (gAd) receptor. Thus, we examined the signaling, metabolic and functional effects of adiponectin and gAd on the heart. Adiponectin promotes a shift from fatty acid oxidation towards glucose oxidation, while gAd elicits the converse effect. Although the metabolic effects of adiponectin and gAd are not associated with changes in AMPK activity, adiponectin and gAd increase and decrease Akt-dependent insulin signalling, respectively. Importantly, the metabolic actions of gAd do not alter cardiac function under aerobic conditions, but are associated with an impaired contractile recovery in reperfused-ischemic hearts.

In summary, this thesis delineates the molecular basis of myocardial AMPK regulation, in response to ischemia, insulin, adiponectin and gAd. Furthermore, two novel AMPKKs were purified that may play an integral role in coordinating AMPK signaling.

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List of Symbols

α: alpha

β: beta

γ: gamma

°C: degrees Celsius

g: relative centrifugal force

g: gram

h: hour

kDa: kilodalton

μg: microgram

μL: microliter

μM: micromolar

μm: micron; micrometer

μU: microunit

mg: milligram

mL: milliliter

mM: millimolar

min: minutes

nmol: nanomole

%: percent

pmol: picomole

U: unit

List of Abbreviations

A.U.: arbitrary units

ACC: acetyl-CoA carboxylase

AICAR: 5-aminoimidazole-4-carboxamide riboside

Akt: protein kinase B

AMP: adenosine monophosphate

AMPK: 5'AMP-activated protein kinase

AMPKK: 5'AMP-activated protein kinase kinase

ANOVA: analysis of variance

ATP: adenosine triphosphate

BSA: bovine serum albumin

CoA: coenzyme A

CD36: fatty acid translocase

cDNA: DNA complementary to messenger RNA

CPT-I: carnitinepalmitoyl-transferase I

Cr: creatine

DNA: deoxyribonucleic acid

DEAE: diethylaminoethyl

DTT: dithiothreitol

EDTA: ethylenediamine tetra-acetic acid

GIK: glucose-insulin-potassium

GLUT: glucose transporter

FABPpm: plasma membrane fatty acid binding protein

FATP: fatty acid transport protein

FGF: fibroblast growth factor

HEPES: 4-(2-hydroxyethyl)-piperazine-1-ethane sulphonic acid

HMW: high molecular weight

IPTG: isopropyl-β-D-thiogalactopyranoside

mmHg: millimeters of mercury

MI: myocardial infarction

PCr: phosphocreatine

PCR: polymerase chain reaction

PEG: polyethylene glycol

PFK-1: phosphofructokinase-1

PFK-2: phosphofructokinase-2

PI3K: phosphatidylinositol-3-kinase

PVDF: polyvinylidene fluoride

RNA: ribonucleic acid

RPP: rate pressure product

SDS-PAGE: sodium dodecylsulphate polyacrylamide gel electrophoresis

SEM: standard error of the mean

VEGF: vascular endothelial growth factor

wt: weight

Chapter 1

INTRODUCTION

1.1 Introduction

5'AMP-activated protein kinase (AMPK) is a member of the sucrose non-fermenting 1 (SNF1) family of Ser/Thr protein kinases that is conserved in plants, yeast and metazoa [1]. AMPK is a heterotrimeric protein complex that is comprised of a catalytic subunit (α) and two non-catalytic subunits (β and γ) [2-4]. The α, β and γ subunits of AMPK are the mammalian orthologues of the *S. cerevisiae* proteins Snf1 [5, 6], Sip1/Sip2/Gal83 [3], and Snf4 [3], respectively, which combine to form the heterotrimeric protein kinase SNF1 [1].

AMPK was first detected in 1973, as an inhibitory HMG-CoA reductase kinase [7] and as an inhibitory acetyl-CoA carboxylase (ACC) kinase [8]. Later, it was found that each of these kinases is stimulated by AMP [9, 10]. Subsequent purification of the HMG-CoA reductase kinase and the ACC kinase revealed that these kinases were identical, and hence, was renamed AMPK [11]. AMPK has since been extensively studied and is known to be regulated by heterotrimer formation, positive allosterism, and phosphorylation [1].

Over the last three decades it has become clear that AMPK plays an important role in the coordination of myocardial glucose and fatty acid metabolism [12]. The importance of metabolic regulation by AMPK is especially pertinent in the ischemic and post-ischemic heart, where the resultant metabolic changes have been shown to contribute to impaired contractile function [13, 14].

In addition, AMPK signaling is targeted by the hormones insulin and adiponectin; and thus modulation of AMPK signaling is integral to the coordination of whole body metabolism.

1.2 Structural Features of AMPK

1.2.1 *AMPK α Subunit*

The catalytic activity of AMPK is mediated by the α subunit. Two isoforms of the AMPK catalytic subunit (α_1 and α_2) have been cloned from human [15, 16] and rat [5, 6] cDNA libraries. α_1 and α_2 are encoded by separate genes (*PRKAA1* and *PRKAA2*, respectively) that have been mapped by fluorescence *in situ* hybridization to the human chromosomes 5p12 and 1p31, respectively [17, 18]. Transcripts for α_1 and α_2 have a differential tissue distribution, as detected by Northern blot analyses [16, 18]; α_1 is widely expressed among various tissues [16], whereas α_2 is most abundantly expressed in skeletal muscle [16, 18].

The predicted 63 kDa α_1 and 62 kDa α_2 polypeptides contain an N-terminal kinase domain [5] and a C-terminal regulatory region [19, 20]. The domain structure and sequence alignment of rat α_1 and α_2 are depicted in Figure 1.1. The N-terminal kinase domains of α_1 and α_2 are highly similar [16], and are conserved with the kinase domain of Snf1 [5, 6]. In contrast, the C-terminal

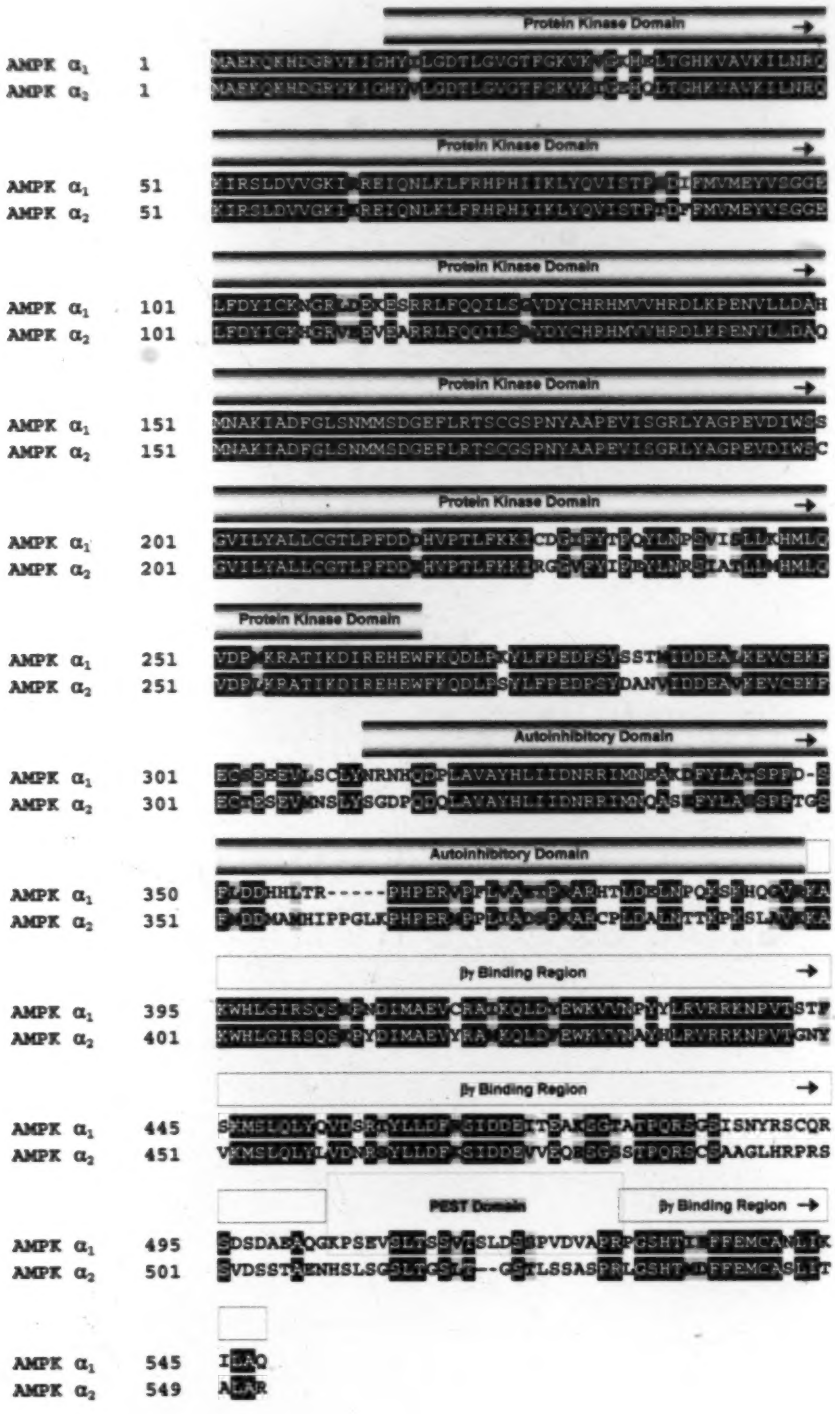
regulatory regions of α_1 and α_2 display significant sequence divergence [16], and are weakly homologous to the C-terminal regulatory region of Snf1 [1]. Structure-function studies have elucidated the presence of distinct regulatory domains within the C terminus [19, 20]. Specifically, an autoinhibitory domain has been mapped to amino acids 313 to 392 of rat α_1 [20]. In addition, the rat α_1 C-terminal residues, 393 to 548, have been found to be essential for $\beta\gamma$ binding and thus heterotrimer formation [20]. Although it is uncertain whether α_2 also contains autoinhibitory and $\beta\gamma$ binding domains, the aligned corresponding regions of α_2 display considerable homology with α_1 [20]. Moreover, the domain architecture of α_1 is conserved in Snf1; the C terminus also contains an autoinhibitory domain [20] and binding regions for the β and γ subunits [21]. Potentially, the sequence divergence between the C termini of α_1 and α_2 may afford a differential regulation of their respective activities and/or localization and this possibility remains to be explored. Of note, α_2 but not α_1 has been shown to localize to the nucleus; however, α_2 does not contain a known conserved nuclear localization signal [22].

Bioinformatic analysis also identified a putative PEST motif, a proline-, glutamatic acid-, serine- and threonine-rich region that regulates proteolysis [23, 24], within α_1 but not α_2 [20]. The PEST motif spans residues 504 to 527 of rat α_1 [20]. Interestingly, although the PEST motif has not been directly demonstrated

to regulate proteolysis of α_1, the half-life of α_1 has been shown to increase when the amino acids 313 to 548 are removed [20].

Figure 1.1 Domain Structure and Sequence Alignment of α_1 and α_2 AMPK Subunits

Domain structure and sequence alignment of rat α_1 and α_2 AMPK primary amino acid sequences (GenBank accession: NP_062015 and A53621, respectively). Sequences were aligned using the Clustal W algorithm [25], at the San Diego Supercomputer Biology Workbench (http://workbench.sdsc.edu). Identical residues (shaded black) and similar residues (shaded gray) are highlighted.


Protein Kinase Domain
AMPK α1 1
AMPK α2 1
Protein Kinase Domain
AMPK α1 51
AMPK α2 51
Protein Kinase Domain
AMPK α1 101
AMPK α2 101
Protein Kinase Domain
AMPK α1 151
AMPK α2 151
Protein Kinase Domain
AMPK α1 201
AMPK α2 201
Protein Kinase Domain
AMPK α1 251
AMPK α2 251
Autoinhibitory Domain
AMPK α1 301
AMPK α2 301
Autoinhibitory Domain
AMPK α1 350
AMPK α2 351
βγ Binding Region
AMPK α1 395
AMPK α2 401
βγ Binding Region
AMPK α1 445
AMPK α2 451
PEST Domain
βγ Binding Region
AMPK α1 495
AMPK α2 501
AMPK α1 545
AMPK α2 549

1.2.2 *AMPK β Subunit*

The β subunit was first identified as a 38 to 40 kDa protein that co-purified with the AMPK α subunit [2-4]. Initially, the β subunit was hypothesized to function as a scaffold [26]; however, recent evidence now suggests that the β subunit also regulates the localization of AMPK [27-29] and is critical for a kinase-independent function of AMPK [28].

In *S. cerevisiae* three isoforms of the β subunit are expressed [30], while in mammals, only two isoforms (β_1 and β_2) have been identified thus far [26, 31]. Partial amino acid sequencing of the β_1 subunit enabled the cloning of the β_1 cDNA from a rat liver cDNA library [26], while the human β_2 cDNA was isolated from a yeast two-hybrid screen with α_2 as the bait [31]. The human β_1 gene (*PRKAB1*) has been mapped to chromosome 12q24 by fluorescence *in situ* hybridization [17], and the human β_2 gene (*PRKAB2*) has been assigned to chromosome 1q21 [32]. The β_1 transcript is widely distributed among both rat and human tissues, and is most abundant in heart and kidney [26, 31]. The β_2 transcript is also widely expressed, but transcripts are most abundant in heart and skeletal muscle, with very low amounts detectable in kidney [31].

The domain structure and sequence alignment of rat β_1 and β_2 are depicted in Figure 1.2. The N-terminal regions of the β_1 and β_2 isoforms do not exhibit significant similarity [31]. However, both isoforms contain a consensus

N-myristoylation sequence [27]. Mass spectrometric analysis of rat β_1 revealed that the N terminus is myristoylated [33] and abolition of the N-myristoylation site by mutagenesis results in a redistribution of the β_1 subunit from cytoplasmic extranuclear particulate sites to cytoplasmic extranuclear nonparticulate regions within the cell [27]. Co-localization experiments have attempted to define the precise localization of the β_1 subunit within the cell [27], but to date, the identity of the cytoplasmic particulate sites remains unknown; however, the mitochondria and endoplasmic reticulum have been excluded as possible subcellular locations for the β_1 subunit [27].

The two isoforms of the β subunit are highly identical, especially within the C-terminal two-thirds of the protein (Figure 1.2) [31]. This region also displays the greatest homology with the SNF1 β subunits [26, 31]. Importantly, two structurally and functionally conserved domains are situated within this region: an αγ binding domain and a glycogen binding domain (Figure 1.2) [34]. The αγ binding domain of the AMPK β subunit is structurally conserved with the "association with SNF1 complex" (ASC) domain [34] found in the three SNF1 β subunits [30]. Unlike the ASC domain, which only binds the SNF1 γ subunit, Snf4 [21], the αγ binding domain interacts with the γ and α subunits [28]. Based on homology with the ASC domain, the mammalian αγ binding domain is predicted to span residues 203-270 of the rat β_1 subunit [31]. The precise residues

that mediate α and γ binding are not known, however experiments with β_1 truncation mutants have revealed that residues 257-270 are critical for both α and γ interactions [28].

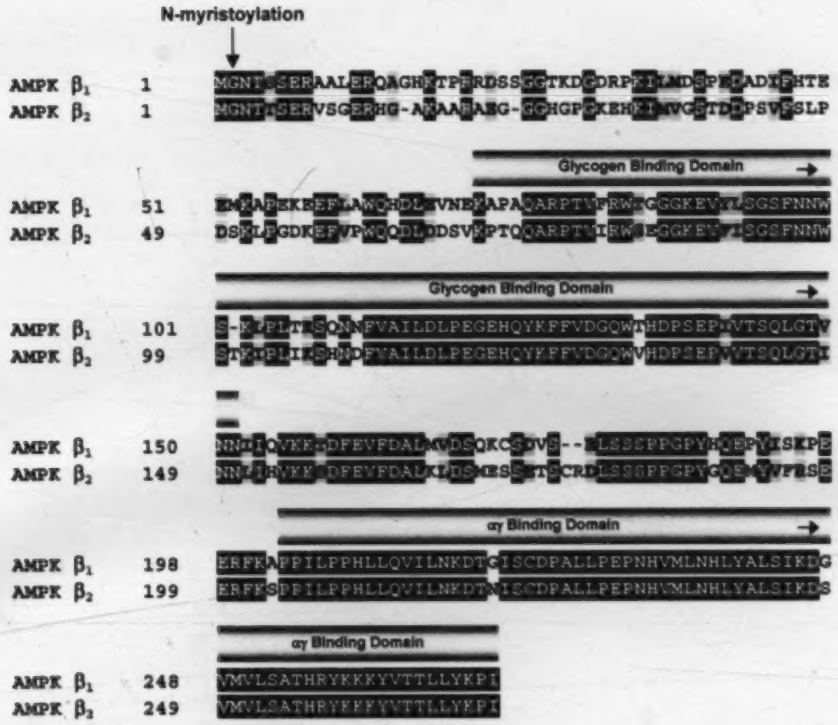
In contrast to the αγ binding domain, the glycogen binding domain is not essential for heterotrimer formation [28]. The glycogen binding domain, or N-isoamylase domain, is found not only in the AMPK and SNF1 β subunits but is also present in glycogen branching enzymes [28, 29]. The domain is predicted to span residues 72 to 151 of rat β_1, and it has been demonstrated that this domain binds glycogen [29]. *In vitro*, both purified rat liver AMPK and a recombinant β_1 glycogen binding domain interact with glycogen [29]. However, purified AMPK has a greater affinity for glycogen than does the β_1 glycogen binding domain [29]. Intriguingly, binding of AMPK to glycogen has no effect on the catalytic activity of AMPK [29].

A recent study has revealed an additional role for the glycogen binding domain; this domain is essential for the accumulation of glycogen-containing inclusion bodies in CCL13 cells, a phenomenon that arises when the AMPK heterotrimer is overexpressed [28]. When mutant β_1 constructs, that lack all or part of the glycogen binding domain, are co-expressed with α_1 and γ_1, the inclusion bodies are no longer prevalent [28]. In addition, the catalytic activity of AMPK heterotrimers is not altered when a β_1 subunit that lacks a portion of the

glycogen binding domain is expressed [28]. Furthermore, the catalytic activity of AMPK is not required for the accumulation of the inclusion bodies [28]. Thus, while the glycogen binding domain is essential for glycogen binding and the formation of large glycogen particles, these effects do not alter or require the catalytic activity of AMPK [28]. Hence, the glycogen binding domain is required for a kinase-independent function of AMPK [28].

Figure 1.2 Domain Structure and Sequence Alignment of β_1 and β_2 AMPK Subunits

Domain structure and sequence alignment of rat β_1 and β_2 AMPK primary amino acid sequences (GenBank accession: NP_114182 and NP_072149, respectively). Sequences were aligned using the Clustal W algorithm [25], at the San Diego Supercomputer Biology Workbench (http://workbench.sdsc.edu). Identical residues (shaded black) and similar residues (shaded gray) are highlighted.


N-myristoylation
AMPK β1 1
AMPK β2 1
Glycogen Binding Domain
AMPK β1 51
AMPK β2 49
Glycogen Binding Domain
AMPK β1 101
AMPK β2 99
αγ Binding Domain
AMPK β1 198
AMPK β2 199
αγ Binding Domain
AMPK β1 248
AMPK β2 249

1.2.3 *AMPK γ Subunit*

The third member of the AMPK heterotrimer, the γ subunit, was also identified as a polypeptide that co-purified with the α catalytic subunit [2-4]. Three mammalian γ isoforms (γ_1, γ_2, and γ_3) have been cloned, and exhibit homology with the only SNF1 γ subunit, Snf4 [26, 35]. Each γ isoform is encoded by separate genes, *PRKAG1*, *PRKAG2* and *PRKAG3*, which have been mapped to human chromosomes 12q13.1 [17], 7q36 [36], and 2q36.1 [37], respectively. Northern blot analyses revealed that γ_1 and γ_2 are widely expressed [26, 35] and are most abundant in the heart, whereas γ_3 is primarily expressed in skeletal muscle [35].

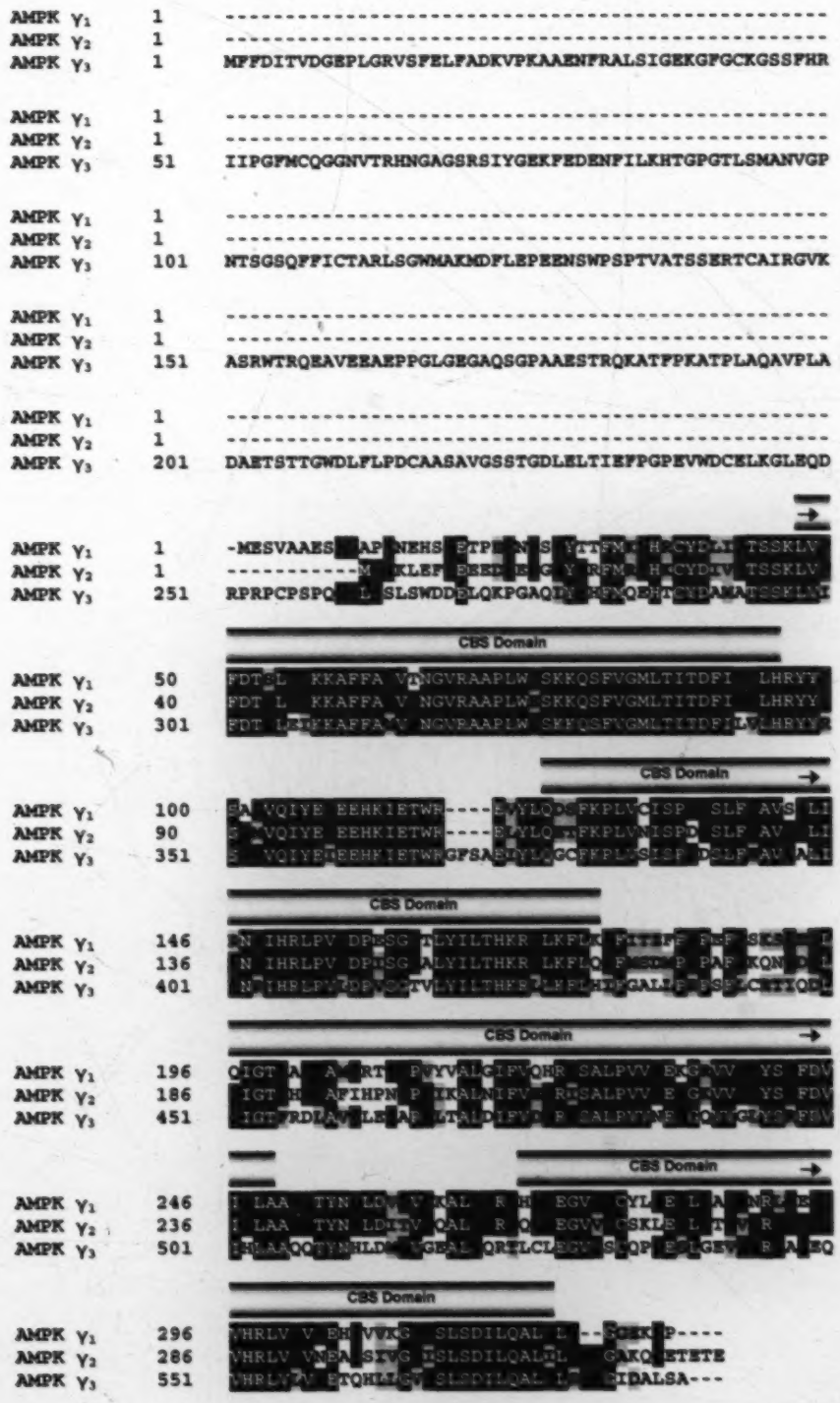
Each of the three γ isoforms contains four cystathionine-β-synthase (CBS) domains (Figure 1.3), which are also conserved in Snf4 [34]. Several lines of evidence suggest that the CBS domains enable the γ subunit to bind the allosteric effectors of AMPK, AMP and ATP. Cheung *et al.* provided the first evidence for the participation of the γ subunit in allosteric control by demonstrating that the γ subunit can be photoaffinity labeled with the AMP analogue 8-azido-AMP [35]. Binding and structural modeling studies have further demonstrated that binding of AMP and ATP is mediated by the CBS domains [38, 39]. Specifically, the folding of two tandem CBS domains forms a pocket for AMP or ATP binding [39]. In addition, Scott *et al.* demonstrated that each pair of CBS domains binds

one molecule of AMP or one molecule of ATP [38]. Furthermore, it has been shown that each γ isoform imparts different degrees of AMP-sensitivity to the AMPK heterotrimer [35]. Thus, it is evident that the γ subunit plays an important role in binding the allosteric effectors of AMPK.

Although, yeast two-hybrid experiments have demonstrated that each γ isoform strongly interacts with the two β isoforms, and that γ_1 interacts with both α isoforms [35], the corresponding regions within the γ subunit that mediate these interactions have not been delineated.

Figure 1.3 Domain Structure and Sequence Alignment of γ_1, γ_2, and γ_3 AMPK Subunits

Domain structure and sequence alignment of rat γ_1, γ_2, and γ_3 AMPK primary amino acid sequences (GenBank accession: P80385, AAQ55225, XP_237293). Sequences were aligned using the Clustal W algorithm [25], at the San Diego Supercomputer Biology Workbench (http://workbench.sdsc.edu). Identical and similar residues conserved among the three isoforms are shaded black and light gray, respectively. Identical residues conserved between two of the isoforms are shaded dark gray.


AMPK γ1 1
AMPK γ2 1
AMPK γ3 1 MFFDITVDGEPLGRVSFELFADKVPKAAENFRALSIGEKGFGCKGSSFHR
AMPK γ1 1
AMPK γ2 1
AMPK γ3 51 IIPGFMCQGGNVTRHNGAGSRSIYGEKFEDENFILKHTGPGTLSMANVGP
AMPK γ1 1
AMPK γ2 1
AMPK γ3 101 NTSGSQFFICTARLSGWMAKMDFLEPEENSWPSPTVATSSERTCAIRGVK
AMPK γ1 1
AMPK γ2 1
AMPK γ3 151 ASRWTRQEAVEEAEPPGLGEGAQSGPAAESTRQKATFPKATPLAQAVPLA
AMPK γ1 1
AMPK γ2 1
AMPK γ3 201 DAETSTTGWDLFLPDCAASAVGSSTGDLELTIEFPGPEVWDCELKGLEQD
CBS Domain
CBS Domain
CBS Domain
CBS Domain
CBS Domain
CBS Domain

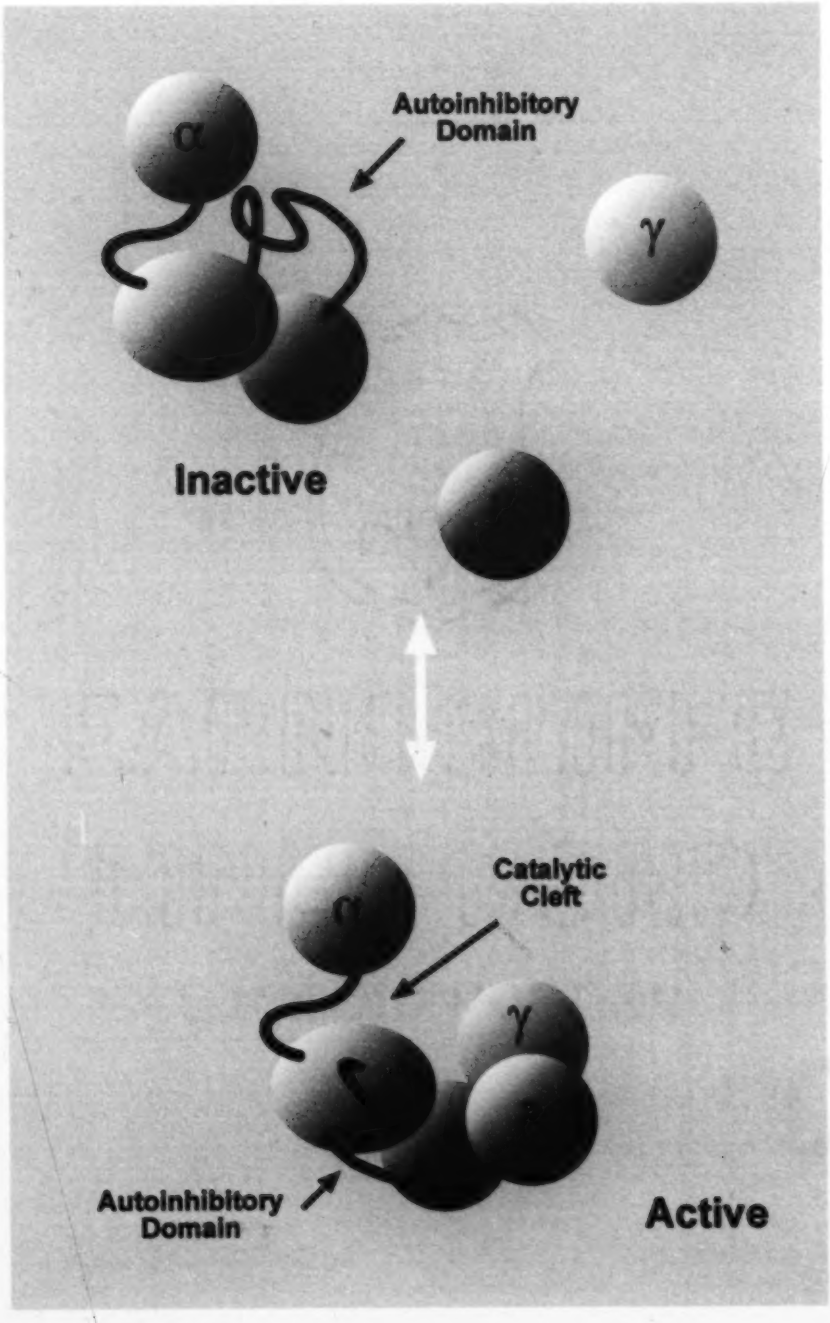
1.2.4 *AMPK Heterotrimer*

At present, it is unknown whether individual AMPK subunits are always associated in a complex. However, it is evident that the αβγ heterotrimeric complex is more active than the individual α subunit [19]. It is postulated that in the absence of interactions with the β and γ subunits, the autoinhibitory domain obstructs the catalytic cleft, and thereby inhibits the catalytic activity of the α subunit (Figure 1.4) [20]. It is also hypothesized that binding of the β and γ subunits to the α subunit causes a conformational change within the α subunit, which repositions the autoinhibitory domain away from the catalytic cleft and relieves the intrasteric inhibition (Figure 1.4) [20].

Although multiple isoforms of each subunit have been cloned, the molecular determinants responsible for specifying isoform composition of the AMPK heterotrimer are unknown. Data from immunoprecipitation experiments suggest that α_2 and γ_1 AMPK complexes constitute ~70% and ~85%, respectively, of the total AMPK activity in the heart [35]. Since α_1 and α_2 localize to different subcellular regions within the cell [22], the functional relevance of the isoform composition of AMPK heterotrimers warrants investigation.

Figure 1.4 Model for the Relief of Intrasteric Inhibition of the AMPK α Subunit by Inter-Protein Interactions with the β and γ Subunits

The model depicted was adapted from the model proposed by Crute *et al.* [20]. The AMPK α subunit is subject to intrasteric inhibition by the autoinhibitory domain and is inactive. Binding of the β and γ subunits to the C terminus of the α subunit induces a conformational change, which repositions the autoinhibitory domain away from the catalytic cleft, and renders the α subunit more active.


Autoinhibitory Domain
α
γ
Inactive
Catalytic Cleft
α
γ
Autoinhibitory Domain
Active

1.3 Regulation of AMPK

1.3.1 *Allosteric Control of AMPK*

Allosterism was the first regulatory mechanism identified for AMPK. In 1980, Yeh *et al.* showed that the activity of ACC kinase, now recognized as AMPK, is stimulated by AMP *in vitro* [10]. Additionally, Yeh *et al.* revealed that high concentrations of ATP inhibit AMPK activity, and antagonize the stimulatory effects of AMP [10]. Thus, AMPK is regulated by changes in the ratio of AMP:ATP.

After the two isoforms of the catalytic subunit were cloned, Salt et al. determined *in vitro* that the activities of α_1 and α_2 liver AMPK complexes are differentially activated by AMP [22]. First, α_2 complexes are activated to a greater degree by AMP than α_1 complexes (5.5-fold and 1.7-fold, respectively) [22]. Second, the half-maximal activation ($A_{0.5}$) of α_1 complexes for AMP is 12 μM whereas the $A_{0.5}$ of α_2 complexes for AMP is 22 μM [22]. Thus, α_2 AMPK complexes are activated by higher concentrations of AMP and are activated to a greater extent than α_1 AMPK complexes.

Consistent with their role in AMP binding, the γ isoforms also confer differential AMP sensitivity. Independent of the α isoform, γ_2 AMPK complexes are stimulated to the greatest extent by AMP, whereas γ_3 AMPK complexes are activated the least by AMP [35].

Recently, the dependency of heart AMPK on cytosolic AMP levels was examined *in vivo* [40]. In this study, it was found that the $A_{0.5}$ of heart AMPK for AMP is 1.8 μM (the $A_{0.5}$ for AMP was determined in hearts with ATP concentrations greater than 7 mM) [40]. Thus, when compared to the $A_{0.5}$ of α_1 or α_2 AMPK (as determined *in vitro* with 0.2 mM ATP) [22], these data suggest that AMPK is more sensitive to changes in AMP levels and less sensitive to ATP antagonism *in vivo*, than purified liver α_1 or α_2 AMPK .

In 1998, an additional allosteric regulator of AMPK was identified. Consistent with its regulation by high energy phosphates, AMPK was found to be inhibited, *in vitro*, by phosphocreatine (PCr) [41]. Furthermore, the inhibitory effect of PCr was antagonized by creatine (Cr) [41]. Thus, AMPK is regulated in response to changes in the ratios of both AMP:ATP and Cr:PCr.

In accordance with the *in vitro* regulation of AMPK by the ratios of AMP:ATP and Cr:PCr, numerous studies have shown that AMPK activity is stimulated in various tissues by treatments that increase the ratios of AMP:ATP and/or Cr:PCr (Table 1.1) [42-44]. However, cardiac AMPK activity does not strictly correlate with the AMP:ATP ratio [45]. During the course of ischemia, AMPK is initially activated in parallel with increases in the ratios of AMP:ATP and Cr:PCr; however, as the ratio of AMP:ATP further increases with continued ischemia, the activity of AMPK declines [45]. Similarly, in other cell types,

AMPK has been shown to be stimulated by metformin, and hyperosmotic stress without concomitant changes in the AMP:ATP ratio (Table 1.1) [46, 47]. Thus, the notion of additional mechanisms for the regulation of AMPK activity is supported.

Table 1.1 Treatments that activate AMPK

Treatments that stimulate AMPK in various tissues and/or cell type are shown. * denotes treatments, which do not affect the cellular AMP:ATP ratio.

Treatment	Tissue/Cell Type
5-aminoimidazole-4-carboxamide riboside (AICAR)	many tissues/cell types including: adipose [48, 49], endothelial cells [50], fibroblasts [51], hepatocyte [52], muscle [53], neuron [54], pancreatic islet [44]
antimycin	Fao hepatoma cells [55], fibroblasts [51], heart [56]
arsenite	hepatocytes [57]
azide	Fao hepatoma cells [55], fibroblasts [51]
dinitrophenol	Fao hepatoma cells [55], muscle [58]
oligomycin	heart [56], hepatocytes [59], monocytes [60]
anoxia, hypoxia	heart [45] , monocytes [60], muscle [58]
ischemia	heart [13]
contraction	muscle [61], cardiomyocytes [62]
exercise	heart [63], liver [64], muscle [65]
low glucose	muscle [66], pancreatic β-cells [44]
hydrogen peroxide	heart [13], muscle [67] , NIH-3T3 cells [68]
peroxynitrite*	endothelial cells [69]
heat shock	hepatocytes [57]
hyperosmotic stress*	muscle [58, 70]
adiponectin	liver [71], muscle [71]
globular domain of adiponectin	adipocytes [72], muscle [71]
ghrelin	hypothalamus [73]
isoproterenol	adipose [74]
leptin	muscle [75]
metformin*, phenformin*	liver [76, 77], muscle [76, 78], neonatal cardiomyocytes [79]
pioglitazone, rosiglitazone	adipose [80], liver [77, 80], H-2K^b cells [46]

1.3.2 *Regulation of AMPK by Phosphorylation*

In 1978, it was discovered that AMPK is positively regulated by phosphorylation [81]. Almost two decades later, the major site phosphorylated by the AMPK kinase (AMPKK) was identified as Thr 172, which resides within the activation loop of the kinase domain in the α catalytic subunit [82]. Mutation of Thr 172 to Asp (T172D), results in AMPK complexes, which are 40 to 50% more active than wildtype α_1 or α_2 AMPK complexes [83]. In addition to increasing the activity of AMPK, producing a T172D mutation also alters the AMP-sensitivity of the AMPK heterotrimer. Interestingly, the T172D mutation increases the $A_{0.5}$ of α_1 and α_2 AMPK complexes by AMP to 43 ± 15 and 57 ± 19 μM, respectively [83]. Strikingly, AMP stimulates α_1 and α_2 T172D complexes by 62- and 44-fold, respectively [83]. Thus, the phosphomimetic T172D mutation, requires a higher concentration of AMP for activation, but is also activated to greater extent by AMP.

Although AMPKK was purified over 1000-fold from liver in 1996 [82], the identity of AMPKK remained elusive for several years. The impetus which led to the identification of AMPKK arose from two independent studies, which systematically identified protein complexes in *S. cerevisiae* [84, 85]. In particular, polymerase alpha kinase 1 (Pak1) was found to interact with Snf4 and Snf1 [85], and the protein kinase Tos3 was also found to interact with Snf4 [84].

Subsequent studies demonstrated that Pak1 and Tos3 phosphorylate and activate Snf1 *in vitro* [86, 87]. However, neither the *pak1*Δ or *tos3*Δ mutants exhibited the phenotypic characteristics of *snf1*Δ mutants [87]. As the double *pak1*Δ *tos3*Δ mutant also did not exhibit the *snf1*Δ phenotype, it was proposed that an additional functionally redundant protein kinase exists [87]. Elm1 was an obvious candidate, as it had been previously identified as a kinase with homology to both Pak1 and Tos3 [88]. Similar to Pak1 and Tos3, Elm1 phosphorylated Snf1 *in vitro* [87, 89]. Furthermore, the triple *pak1*Δ *tos3*Δ *elm1*Δ mutant resembled the *snf1*Δ phenotype. Hence, the three kinases Pak1, Tos3 and Elm1 function as Snf1 kinase kinases. Intriguingly, it was also found that all three of these kinases phosphorylate and activate AMPK *in vitro*. Thus, mammalian Pak1/Elm1/Tos3 orthologues may also function as AMPKKs. Bioinformatic analysis demonstrated that the catalytic domains of Pak1, Tos3 and Elm1 are most similar to mammalian Ca^{2+}/calmodulin-dependent protein kinase kinase and also display homology to the mammalian protein kinase LKB1/STK11 [87]. Thus, these kinases represent putative AMPKKs.

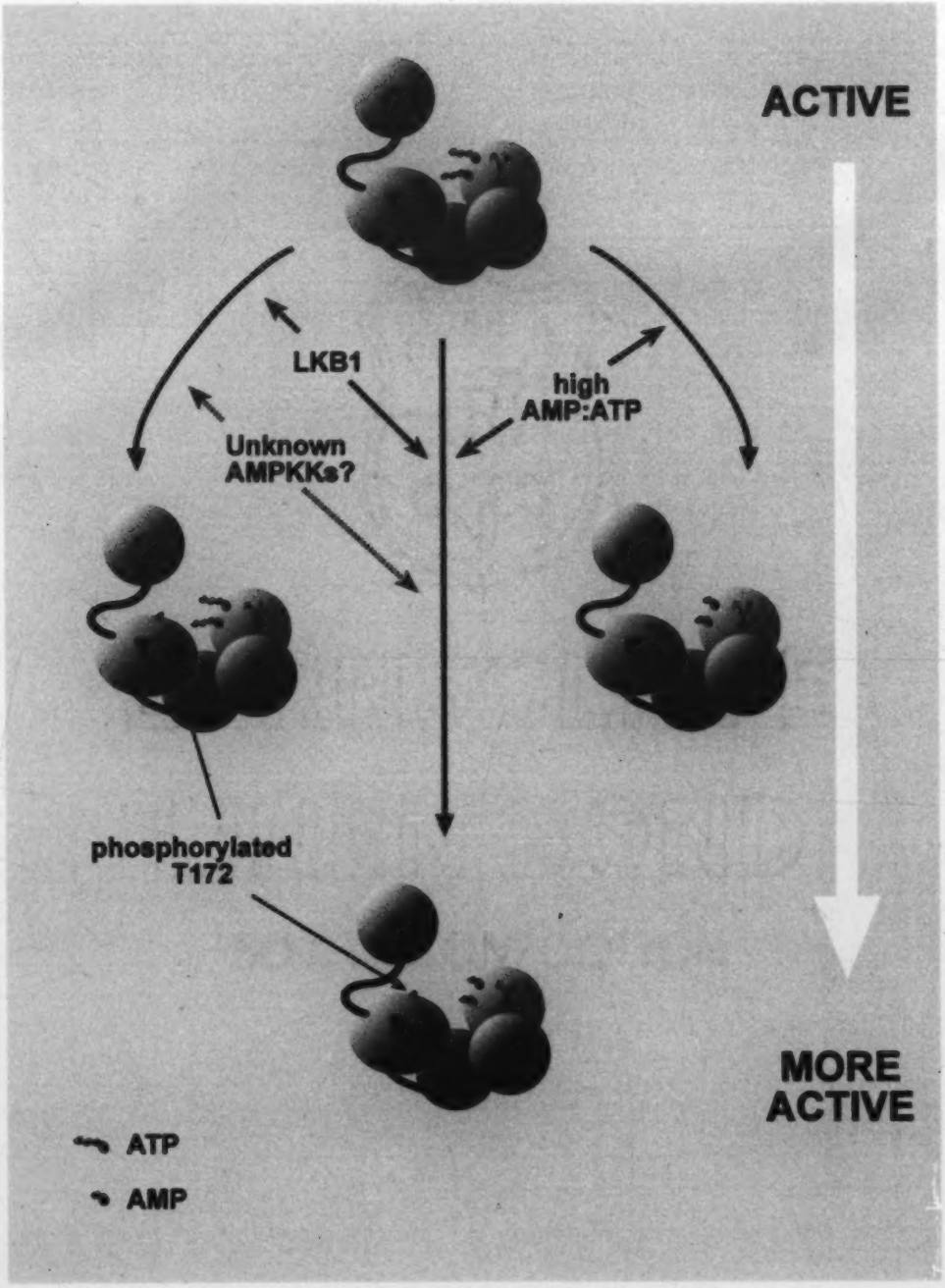
Collectively, subsequent studies from independent groups provided further and strong support for the notion of LKB1 being the elusive mammalian AMPKK. First, recombinant LKB1 was shown to phosphorylate and activate recombinant AMPK heterotrimers *in vitro* [87, 90-92]. Second, immunoblot

analysis of fractions obtained from chromatographic separations of AMPKK revealed that LKB1 co-purifies with liver AMPKK [90, 91]. Third, HeLa cells, which do not express LKB1, and murine embryonic fibroblasts (MEFs), derived from LKB1 knockout mice, display reduced stimulation of AMPK T172 phosphorylation in response to 5-aminoimidazole-4-carboxamide riboside (AICAR), phenformin or H_2O_2 [91, 92]. Furthermore, expression of wild-type, but not kinase dead LKB1, in HeLa cells and LKB-deficient MEFs restores the stimulation of AMPK T172 phosphorylation by AICAR, phenformin and H_2O_2 [91, 92].

Although it is clear that LKB1 functions as an AMPKK, it is unclear whether LKB1 is the sole AMPKK in mammalian cells. As stated earlier, three distinct protein kinases serve as Snf1 kinase kinases in *S. cervisiae* [87, 89]. Moreover, LKB1 is not activated in conjunction with AMPK [90]. Thus it is possible that additional distinct AMPKKs may also exist in mammals.

Figure 1.5 Model of the Regulation of AMPK by Allosterism and Phosphorylation

The catalytic activity of AMPK is allosterically activated by increases in the AMP:ATP ratio or by LKB1-mediated phosphorylation of T172. As discussed in the text, AMPK may be phosphorylated by other AMPKKs. The combined actions of positive allosterism and phosphorylation results in a highly active AMPK.


ACTIVE
LKB1
high
AMP:ATP
Unknown
AMPKKs?
phosphorylated
T172
MORE
ACTIVE
ATP
AMP

1.4 Modulation of Myocardial Metabolism by AMPK

1.4.1 *AMPK Control of Glucose and Fatty Acid Uptake*

The uptake of glucose and fatty acids into the myocyte is mediated by members of the GLUT family of facilitative transporters, and by fatty acid transporters and passive diffusion, respectively. In particular, two GLUT transporters with a high affinity for glucose, GLUT1 and GLUT4, are important in the transport of glucose into the myocyte [93-95]. Of note, GLUT4 is more highly expressed in the heart than GLUT1 [96, 97]. To date, four fatty acid transporters have been identified in the heart and include fatty acid translocase or CD36, plasma membrane fatty acid binding protein (FABPpm), fatty acid transport protein 1 (FATP1) and fatty acid transport protein 6 (FATP6) [98, 99]. At present, the relative roles and regulation of the individual fatty acid transporters are not well understood. However, CD36 is important for fatty acid utilization as myocardial fatty acid oxidation is reduced in CD36-null mice [100, 101].

Recently, it has become evident that GLUT1, GLUT4 and CD36 are regulated by AMPK. GLUT1, GLUT4 and CD36 are localized within the sarcolemmal membrane and within intracellular vesicles [93, 94]. Contraction, mitochondrial uncouplers, and ischemia promote glucose uptake by stimulating the translocation of GLUT1 and GLUT4 to the sarcolemmal membrane [94, 102,

103]. Similarly, translocation of CD36 and fatty acid uptake are stimulated by contraction and mitochondrial uncouplers [104]. These processes are not inhibited by wortmannin and thus involve a phosphatidyinositol-3-kinase (PI3K) independent pathway [105-108].

Interestingly, it was demonstrated that activation of AMPK by AICAR treatment stimulates myocardial glucose uptake and GLUT4 translocation [109]. In addition, it was found that ischemia-stimulated glucose uptake is blunted in dominant-negative α_2 AMPK transgenic mouse hearts [110, 111]. Hence, α_2 AMPK stimulates glucose uptake in response to ischemia. Presently, it is unclear whether AMPK also regulates GLUT1 in the heart. However, in other cell types, AICAR treatment or expression of constitutively active AMPK has been shown to activate cell-surface GLUT1 [112, 113].

Luiken *et al.* also established that stimulation of AMPK, by AICAR or dinitrophenol treatment, enhances CD36-mediated fatty acid uptake in myocytes [107]. Moreover, an additive effect on fatty acid uptake was not evident with electrical stimulation and AICAR or dinitrophenol treatment; suggesting that contraction-induced fatty acid uptake is, in part, mediated by increased AMPK signaling [107].

1.4.2 *AMPK Control of Malonyl-CoA Homeostasis*

Although long-chain fatty acids are oxidized within the mitochondria, the inner mitochondrial membrane is impermeable to acyl-CoA, and acyl-CoA must be converted to acyl-carnitine to facilitate transport into the mitochondria [114]. This reaction is catalyzed by carnitine palmitoyl-transferase I (CPT-I) [114]. Since, overexpression of CPT-I or pharmacological inhibition of CPT-I increases and decreases fatty acid oxidation, respectively [115, 116], CPT-I not only facilitates mitochondrial fatty acid uptake, but is also an important modulator of mitochondrial fatty acid oxidation. The activity of CPT-I is inhibited by malonyl-CoA [117], which is synthesized and degraded by the enzymes ACC and malonyl-CoA decarboxylase (MCD), respectively [118, 119]. Notably, both ACC and MCD are regulated by AMPK [120].

As discussed earlier, AMPK was originally purified and identified as an inhibitor of liver ACC activity [11]. Two isoforms of ACC have been cloned, ACCα and ACCβ [121-123]. Transcripts for each ACC isoform exhibit a differential tissue distribution [121, 122]. ACCα is widely expressed, whereas ACCβ is predominantly expressed in heart and skeletal muscle [121-123]. Early studies demonstrated that AMPK phosphorylation of ACCα results in a decreased V_{max} and an increased $A_{0.5}$ for citrate, the allosteric activator of ACC [124]. Furthermore, AMPK phosphorylates ACCα at Ser 79, Ser 1200 and Ser

1215 [124]. Specifically, AMPK-mediated inhibition of ACC activity occurs through phosphorylation of rat ACCα Ser 79 [125]. Importantly, Ser 79 is also conserved in rat ACCβ and corresponds to Ser 218 [126]. Although it has not been directly demonstrated that AMPK phosphorylates ACCβ at Ser 218, AMPK has been shown to phosphorylate both ACCα and ACCβ isolated from rat heart [127].

Due to the existence of multiple ACC and AMPK isoforms, the precise regulation of ACC, and hence malonyl-CoA synthesis, by AMPK has not been fully resolved. In particular, the relative roles of α_1 and α_2 AMPK in regulating ACC are unclear. *In vitro,* neither ACCα nor ACCβ are phosphorylated by α_2 AMPK, but are highly phosphorylated by α_1 AMPK [128]. In contrast, α_2 AMPK but not α_1 AMPK co-purifies with ACCα and ACCβ isolated from rat heart [127].

It has been hypothesized that ACCβ, due to its mitochondrial localization, plays a more significant role than cytosolic ACCα in the regulation of mitochondrial fatty acid oxidation [129]. Consistent with this hypothesis, skeletal muscle fatty acid oxidation is elevated in ACCβ-null mice [130]. In addition, malonyl-CoA levels are drastically reduced in skeletal muscle and hearts from ACCβ-null animals [130].

Malonyl-CoA levels are also regulated by the enzyme MCD, which catalyzes the decarboxylation of malonyl-CoA to generate acetyl-CoA [131].

While some studies have demonstrated that activation of AMPK increases MCD activity [132, 133], other studies have found that MCD activity is unaffected by stimulation of AMPK [134]. Moreover, AMPK does not phosphorylate MCD *in vitro* [134, 135]. However, it was recently shown that constitutively active AMPK increases mitochondria-associated MCD activity and protein abundance [136]. The increase in MCD activity was not associated with an increase in phosphorylation and mitochondria-associated MCD did not co-immunoprecipitate with constitutively active AMPK [136]. Taken together, these data suggest that AMPK does not directly regulate MCD activity, but may indirectly modify the activity of MCD by a mechanism that remains to be defined.

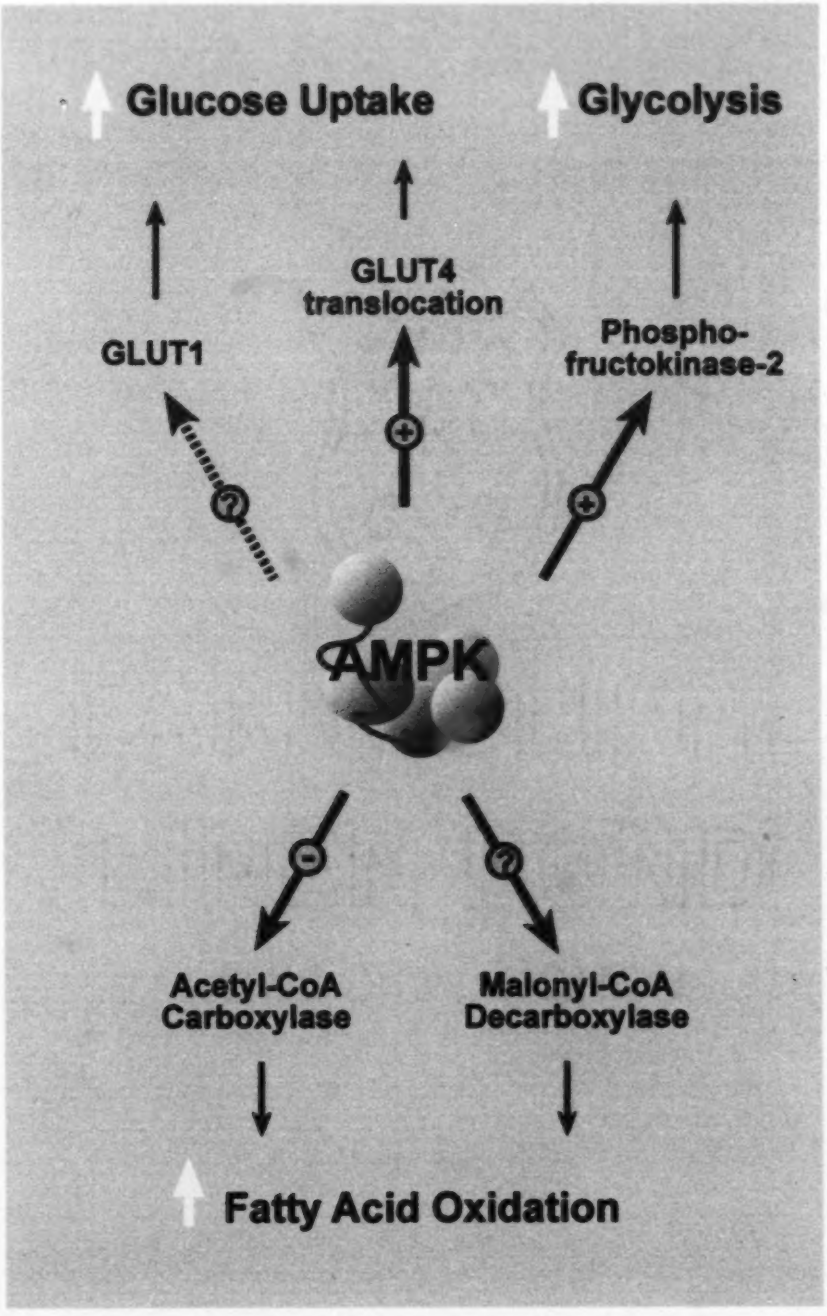
1.4.3 *AMPK Control of Glycolysis*

The heart isoform of phosphofructokinase-2 (PFK-2) is a recently identified metabolic target of AMPK. Heart PFK-2 synthesizes fructose-2,6-bisphosphate [137], an allosteric activator of the glycolytic enzyme, phosphofructokinase-1 (PFK-1) [138]. *In vitro*, AMPK phosphorylates PFK-2 at Ser 466 and thereby increases the activity of PFK-2 [56]. A differential effect on PFK-2 activity was not evident when α_1 or α_2 AMPK was assessed [56]. During ischemia, PFK-2 is activated in conjunction with AMPK [56]. Similarly, in cells treated with AICAR

or oligomycin, PFK-2 Ser 466 phosphorylation is stimulated in parallel with AMPK activation [56]. Furthermore, PFK-2 phosphorylation and activation following oligomycin treatment is abolished when dominant negative α_1 AMPK is expressed [56]. Thus, these data strongly suggest that AMPK stimulates PFK-2 activity and thereby plays a stimulatory role in the regulation of glycolysis.

Figure 1.6 Metabolic Targets of AMPK in the Heart

Depicted are the known metabolic targets and resulting metabolic actions of AMPK in the heart.


Glucose Uptake
Glycolysis
GLUT4
translocation
GLUT1
Phospho-
fructokinase-2
AMPK
Acetyl-CoA
Carboxylase
Malonyl-CoA
Decarboxylase
Fatty Acid Oxidation

1.5 Physiological Roles of Myocardial AMPK

1.5.1 *AMPK Modulation of Myocardial Metabolism During Ischemia*

Under aerobic conditions, the majority of ATP synthesized by the heart is derived from the oxidation of long-chain fatty acids [139]. During ischemia, glycolytic ATP production initially increases [140], while overall oxidative metabolism decreases or is completely inhibited, depending on the availability of oxygen [139]. However, in the presence of residual oxygen, fatty acid oxidation predominates over glucose oxidation [141, 142]. Similarly, upon reperfusion the oxidation of fatty acids prevails over the oxidation of glucose [143].

Since the discovery of AMPK, it has become evident that the initial metabolic changes, which ensue following ischemia or during reperfusion, are in part, mediated by the activation of AMPK. Ischemia induces a dramatic stimulation in the phosphorylation and activation of AMPK [13, 45, 144]. Studies have also demonstrated that increased AMPK activity results in the stimulation of glucose uptake and glycolysis, through the promotion of GLUT4 translocation and PFK-2 activity respectively [56, 109]. Additionally, AMPK activation during ischemia and reperfusion also derepresses fatty acid oxidation by inhibiting ACC production of malonyl-CoA [13].

Importantly, during ischemia and reperfusion, glycolysis is uncoupled from glucose oxidation [145]. The uncoupling results in a net accumulation of

protons [146, 147], which when extruded during reperfusion ultimately lead to Ca^{2+} overload and contractile dysfunction [146]. Metabolic interventions that stimulate glucose oxidation directly, or indirectly by inhibiting fatty acid oxidation have been shown to improve cardiac function in the ischemic and post-ischemic heart [148]. Since AMPK signaling contributes to the metabolic alterations that occur during ischemia and during reperfusion following ischemia, it is important to further understand the mechanisms that regulate AMPK signaling in the heart.

Currently, it is hypothesized that an increase in the AMP:ATP ratio following ischemia stimulates AMPK activity [12]. However, it is unknown whether the increased AMPK phosphorylation also arises from an increased AMPKK/LKB1 activity. Furthermore, the relative roles of positive allosterism and AMPKK in stimulating AMPK during ischemia remain to be defined.

1.5.2 *Crosstalk between Insulin and AMPK Signaling in the Heart*

Insulin, like ischemia, promotes glucose uptake and glycolysis in the heart [149]. However, insulin and ischemia employ different signaling pathways to mediate similar effects on glucose metabolism [150] and this is evident by the combinatorial effect of insulin and ischemia on GLUT4 translocation and glucose uptake. Specifically, insulin stimulates GLUT4 translocation and PFK-2

activation through a PI3K-dependent pathway, while ischemia promotes the identical processes through AMPK [150]. Akt is a known effector for insulin signaling [152] and accordingly Akt has been shown to stimulate GLUT4 translocation in the heart [153]. Furthermore, Akt also phosphorylates heart PFK-2 at Ser 466 [154].

Interestingly, insulin has been demonstrated to inhibit the activity and Thr 172 phosphorylation of AMPK [45, 155-157]. The inhibition of AMPK by insulin occurs in the absence of changes in the AMP:ATP ratio [45]. Additionally, the inhibitory effects of insulin on AMPK are antagonized by fatty acids [158], which also inhibit insulin signaling through Akt [159]. In cardiac myocytes and the heart, overexpression of constitutively active Akt results in decreased AMPK phosphorylation [155]. Thus, crosstalk between insulin signaling and AMPK signaling is mediated by Akt. However, it remains to be resolved whether insulin/Akt directly inhibits AMPK and/or indirectly inhibits AMPK by decreasing the activity of AMPKK.

1.5.3 *Modulation of AMPK Signaling and Myocardial Metabolism by Leptin, Adiponectin and the Globular Domain of Adiponectin*

The adipocyte-derived hormones leptin, adiponectin and the C-terminal globular domain of adiponectin (gAd) have been shown to regulate whole body

energy homeostasis [71, 160-166]. By promoting hepatic glucose output and glucose uptake in muscle, leptin stimulates whole body glucose turnover without affecting plasma glucose levels [167, 168]. In addition, leptin stimulates fatty acid oxidation and depletion of intracellular triacylglycerol stores in heart, muscle, and pancreas [169-171]. Of note, the effects of leptin on peripheral tissue fatty acid metabolism are not accompanied by a decline in plasma free fatty acid levels [169]. Currently, the mechanisms by which leptin regulates glucose and fatty acid metabolism are not completely understood. However, it is evident that stimulation of whole body glucose turnover by leptin is dependent on the central nervous system [167, 168], while leptin-stimulated partitioning of fatty acids to oxidation rather than storage, is mediated by AMPK in muscle [75], but not by AMPK in heart [171].

Through their actions on the brain and peripheral tissues, adiponectin and gAd lower plasma glucose and free fatty acid concentrations, respectively [71, 160-164]. In skeletal muscle, adiponectin and gAd stimulate both glucose uptake and fatty acid oxidation [71]. As AMPK also stimulates glucose uptake and fatty acid oxidation [13, 109, 172, 173], AMPK has emerged as a candidate effector for adiponectin and gAd signaling. In accordance, studies have found that gAd stimulates AMPK activity and Thr 172 phosphorylation in skeletal muscle [71, 162]. Furthermore, expression of a dominant-negative AMPK blunted gAd-

stimulated glucose uptake and fatty acid oxidation in C2C12 cells [71], thereby demonstrating a direct role for AMPK in gAd signaling.

In humans, plasma adiponectin levels are inversely correlated with coronary artery disease [174, 175] and decrease following the onset of myocardial infarction [176]. Conversely, high plasma adiponectin levels are associated with a lower risk of myocardial infarction [177]. Despite the potential protective role of adiponectin in the heart, no studies have investigated the metabolic and functional effects of adiponectin or gAd on the adult heart.

1.6 Thesis Objectives

Although the pivotal role of AMPK in the regulation of myocardial metabolism has previously been established, the mechanisms that modulate AMPK activity during ischemia or mediate insulin signaling crosstalk to AMPK have not been elucidated. In addition, it is presently unclear whether adiponectin or gAd modulates metabolism and/or AMPK signaling in the adult heart. Thus, the major objective of this thesis is to further delineate the mechanisms that regulate AMPK activity in the heart. Specifically, we aim to:

1) Determine whether ischemia stimulates myocardial AMPK by activating the upstream kinase AMPKK/LKB1.

2) Investigate whether insulin inhibits AMPK by depressing the activity of AMPKK in the heart.

3) Elucidate the effects of adiponectin and gAd on AMPK signaling, metabolism and contractile function in the heart.

4) Purify heart AMPKK(s) that are distinct from LKB1.

Through these specific aims, this thesis will provide the first examination of the role of AMPKK in the regulation of myocardial metabolism during ischemia. Further examination of the effects of insulin on AMPKK activity will help define the molecular basis for insulin-mediated inhibition of AMPK signaling in the heart. Characterization of the molecular, cellular and functional effects of adiponectin and gAd in the adult heart will aid in the resolution of the physiological roles of adiponectin and gAd. Purification of LKB1-distinct AMPKK(s) will enable the molecular identification and characterization of additional protein kinase(s) that regulate AMPK in the heart. Together, these studies will advance our current understanding of the molecular and hormonal mechanisms that regulate AMPK in the heart.

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Chapter 2

Myocardial Ischemia Differentially Regulates LKB1 and an Alternate 5′AMP-Activated Protein Kinase Kinase

2.1 Introduction

During myocardial ischemia, the oxidative metabolism of glucose and fatty acids decreases due to the limited availability of oxygen [1]. Consequently, glycolysis initially increases to compensate for the decreased supply of ATP from mitochondrial oxidative metabolism [2]. Although oxidative metabolism is decreased during ischemia [1], fatty acid oxidation predominates over glucose oxidation and hence is the major consumer of residual oxygen [3, 4]. Importantly, the combined preferential oxidation of fatty acids and elevated rates of glycolysis, leads to an increased production of protons and lactate in the ischemic heart, which contribute to cardiac inefficiency and contractile dysfunction [5].

Several lines of evidence implicate 5'AMP-activated protein kinase (AMPK) as an important mediator of the metabolic changes in glycolysis and fatty acid oxidation that arise during and following myocardial ischemia. AMPK is rapidly activated during myocardial ischemia [6-8] and remains high during early reperfusion [6]. Activation of AMPK promotes glucose uptake [9, 10] by stimulating the translocation of GLUT4 transporters to the sarcolemmal membrane [9]. In addition, AMPK indirectly stimulates phosphofructokinase-1 (PFK-1) activity by phosphorylating and activating the heart isoform of PFK-2 [11], which synthesizes the allosteric activator of PFK-1, fructose 2,6-

bisphosphate [12]. Furthermore, AMPK also stimulates myocardial fatty acid oxidation [6] by phosphorylating and inhibiting acetyl-CoA carboxylase (ACC) [13]. The AMPK-mediated inhibition of ACC results in a decreased synthesis of malonyl-CoA [6], a potent inhibitor of mitochondrial fatty acid uptake [14]. Thus, activation of AMPK, during and following ischemia, is associated with enhanced glucose uptake [10], increased PFK-2 activity [11], and diminished ACC activity [6, 13]. These events culminate in an accelerated rate of glycolysis and the preferential oxidation of fatty acids over glucose.

AMPK is a member of an evolutionarily conserved family of serine/threonine protein kinases that function as sensors of the energy-state of the cell [15]. The heterotrimeric kinase is composed of a catalytic subunit (α) and two regulatory subunits (β and γ) [16]. The catalytic activity of AMPK is increased following association of the β and γ subunits with the α subunit [17]. Additionally, AMPK activity is altered by changes in the cellular levels of high-energy phosphates [18]. Specifically, AMPK is allosterically activated by increases in the ratios of AMP:ATP [18] or creatine (Cr):phosphocreatine (PCr) [19]. Although positive allosterism plays an important role in regulating AMPK [18, 19], it is now evident that it is not essential for AMPK activation [20, 21]. Importantly, it has been found that changes in AMPK activity following

ischemia, insulin, metformin, or hyperosmotic stress, do not correlate with the ratio of AMP:ATP [8, 20, 21].

The catalytic activity of AMPK is also governed by the reversible phosphorylation of the residue Thr 172 [22, 23], which is situated within the activation loop of the kinase domain of the AMPK α subunit [22]. AMPK kinase (AMPKK) phosphorylates and activates AMPK [22], while the protein phosphatases PP2A and PP2C dephosphorylate and thereby decrease the activity of AMPK [23]. Although early studies by Hardie's laboratory demonstrated that AMP enhances AMPKK activity *in vitro* [18, 24, 25], Hardie's group recently reported that the activity of a more purified AMPKK is not affected by AMP [26]. Thus, unlike AMPK, AMPKK is not regulated by AMP.

Recently, the protein kinase LKB1 was identified as an AMPKK [26-28]. However, several lines of evidence suggest that other AMPKK isoforms may also exist. Firstly, in *S. cerevisiae* three distinct protein kinases have been identified as activators of the AMPK orthologue, Snf1 [27, 29]. Secondly, in LKB1-deficient cells, the activity of AMPK is mildly reduced whereas the activities of other LKB1-regulated kinases, are nearly abolished [30]. Additionally, the activity of LKB1 is not affected by stimuli that activate AMPK [28, 31], whereas the activity of AMPKK has been found to increase concurrently with AMPK following exercise [32].

Since AMPK is an important mediator of the metabolic sequelae that arise during and following myocardial ischemia, we determined whether ischemia stimulates AMPK via AMPKK and/or LKB1. In addition, we investigated whether positive allosterism is required for the stimulation of AMPK by ischemia.

2.2 Materials and Methods

2.2.1 *Heart Perfusions*

All of the animals used in this study were cared for in accordance with the guidelines provided by the Canadian Council on Animal Care.

Hearts from fed male Sprague-Dawley rats (300 to 350 g) were isolated and perfused as working preparations, as described previously [5, 33]. In one series of perfusions, hearts were subjected to either 60 min of aerobic perfusion or 30 min of aerobic perfusion followed by 30 min of global severe ischemia. The left atrial inflow line and the aortic outflow line were clamped to produce a global severe ischemia [5]. Isolated working hearts were perfused with a modified Krebs-Henseleit solution that contained 2.5 mM Ca^{2+}, 100 μU/mL insulin, 11 mM glucose, and 1.2 mM palmitate bound to 3% defatted bovine serum albumin (Cohn fraction V; Boehringer Mannheim). In a second series of perfusions, hearts were subjected to either 60 min of aerobic perfusion or 30 min

of aerobic perfusion followed by 30 min of mild ischemia. The one-way ball valve technique [34] was modified to produce a controlled sustained mild-ischemia. The one-way ball valve was situated in one tract of a bifurcated aortic outflow line, and was designed to not impede systolic ejection [34]. A backflow controller was placed in the second tract of the aortic outflow line to permit a proportion of ejected perfusate to bypass the one-way ball valve and perfuse the coronary circulation during diastole in a controlled manner. The perfusate was a modified Krebs-Henseleit solution that contained 2.5 mM Ca^{2+}, 100 μU/mL insulin, 5.5 mM D-[5-^{3}H(N)]-glucose, and 1.2 mM palmitate bound to 3% defatted bovine serum albumin (Cohn fraction V; Sigma). As described previously, rates of glycolysis were determined by measuring the rate of production of $^{3}H_2O$ [35].

At the end of the perfusions, all hearts were quickly frozen with Wollenberger clamps cooled to the temperature of liquid N_2. Frozen hearts were ground to a powder using a mortar and pestle cooled to the temperature of liquid N_2. Portions of the frozen powdered tissue were utilized to determine the dry to wet ratio and for biochemical measurements.

2.2.2 *High Energy Phosphate Measurements*

The tissue contents of AMP, ATP, Cr and PCr were determined in neutralized perchloric acid extracts of frozen powdered heart tissue by high-performance liquid chromatography, as described previously [35, 36].

2.2.3 *Protein Extractions*

Frozen powdered heart tissue was homogenized at 4°C in a buffer containing 50 mM Tris-HCl (pH 8 at 4°C), 1 mM ethylenediamine tetra-acetic acid (EDTA), 10% (w/v) glycerol, 0.02% (v/v) Brij-35, 1 mM dithiothreitol (DTT), protease inhibitors (P 8340; Sigma), and phosphatase inhibitors (P 2850 and P 5726; Sigma). The homogenate was cleared by centrifugation at 13000 *g* for 20 min at 4°C. The resulting supernatant was adjusted to contain 120 mM NaCl and 5% (w/v) PEG6000, vortexed gently for 1 h at 4°C and centrifuged at 10000 *g* for 10 min at 4°C. The activities of AMPKK and AMPK were determined from the 5% PEG6000 supernatant and cleared homogenate, respectively. Protein concentrations of the cleared homogenate and 5% PEG6000 supernatant were determined by the method of Bradford [37].

2.2.4 *Immunoblotting*

Equal amounts of cleared homogenate were resolved by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were probed first with one of the following primary antibodies obtained from Cell Signaling Technologies: anti-AMPK, anti-phosphoThr 172 AMPK, anti-phosphoSer 79 ACC, or anti-LKB1. Blots were then probed with a horseradish peroxidase-coupled goat anti-rabbit secondary antibody (Jackson ImmunoResearch). Immunoblots for ACC were conducted using horseradish peroxidase-labeled streptavidin (Kirkegaard and Perry Laboratories). All of the aforementioned antibodies were used at the concentrations recommended by the manufacturer. Blots were developed using enhanced chemiluminescence reagent (Amersham Biosciences). Detected immunocomplexes were scanned using a calibrated densitometer (BioRad) and quantified using Quantity One software (BioRad).

2.2.5 *Recombinant Protein Expression and Purification*

The C-terminal truncation mutant of the AMPK α1 subunit encoding amino acids 1 to 312 (α_{312}) was amplified from the full-length rat α_1 cDNA (a gift from Dr. D. Carling, Imperial College of London) by the polymerase chain reaction. The sense and antisense primers used were: 5′-

GTCGTACCATGGCCGAGAAGCAGAGCACGA-3′ and 5′-CACTAGCTCGAGGTACAGGCAGCTGAGGA-3′, respectively. The amplicon was cloned into pCR2.1-TOPO (Invitrogen) and then subcloned into the bacterial expression vector pET30a using the restriction sites *Nco*I and *Xho*I. The resulting construct (α_{312}-pET30a) encodes α_{312} with an S-tag and His_6-tag at the N-terminus and a His_6-tag at the C-terminus.

The *E. coli* strain BL21(DE3) was transformed with the α_{312}-pET30a construct. Cells were grown to the mid-log phase and were then induced to express α_{312} by incubating the cells for 3 h at 37°C in the presence of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Cells were pelleted by centrifugation at 10000 *g* for 10 min at 4°C. Cells were lysed by freeze-thawing and sonication in 50 mM NaH_2PO_4 (pH 8 at 4°C), 500 mM NaCl, 10% (w/v) glycerol, 0.02% Brij-35, 5 mM β-mercaptoethanol, 5 U/mL benzonase nuclease (Novagen), 100 μg/mL lysozyme (Sigma) and protease inhibitors (P 8849; Sigma). The lysate was cleared by centrifugation at 13000 *g* for 30 min, adjusted to pH 8 at 4°C, and then filtered through a 0.45 μm filter.

Recombinant α_{312} was purified using a Biologic HR chromatography system (BioRad). All of the purification procedures were conducted at 4°C. The cleared lysate was loaded onto a Ni^{2+} chelating sepharose column (Amersham Biosciences) that was equilibrated in buffer A (50 mM NaH_2PO_4 (pH 8 at 4°C),

10% w/v glycerol, 0.02% Brij-35 and 500 mM NaCl). The column was washed with 5 column volumes of buffer A, and α_{312} was eluted using a step gradient of imidazole. Fractions that contained purified α_{312} were pooled and exchanged into buffer B (50 mM Tris (pH 8 at 4°C), 100 mM NaCl, 10% (w/v) glycerol, 0.02% (w/v) Brij-35 and 5 mM β-mercaptoethanol), by dialysis. Purified α_{312} was concentrated by centrifugation using Amicon Ultra (Millipore) centrifugal filters with a molecular weight cutoff of 10 kDa. Protease inhibitors (P 8849; Sigma) and 1 mM DTT were added to the purified α_{312}, which was then stored in aliquots at -80°C. In this study, α_{312} was thawed immediately prior to use in the AMPKK assay.

2.2.6 *AMPK Assay*

The activity of AMPK in homogenates was assayed as described previously [6], except the synthetic AMARA peptide (AMARAASAAALARRR) [38] was used as the substrate. Briefly, 5 μg of homogenate was incubated in a reaction mixture that contained 40 mM HEPES (pH 7), 80 mM NaCl, 8% (w.v) glycerol, 0.8 mM EDTA, 0.8 mM DTT, 5 mM $MgCl_2$, 200 μM ATP, 1 μCi [γ-^{32}P]-ATP, and 0 or 200 μM AMARA. Reactions were initiated by the addition of sample, and were incubated for 10 min at 30°C. The reactions were terminated by spotting an aliquot of each reaction mixture onto a separate P81

phosphocellulose paper (Whatman). The papers were washed 4 times with 500 mL of 1% (v/v) H_3PO_4 and once with 500 mL of acetone. Each paper was air-dried and was then transferred to a vial containing 4 mL of scintillant. The radioactivity was measured with a scintillation counter (Beckman).

2.2.7 *AMPKK Assay*

At the outset of this study, an assay to measure the activity of AMPKK in the heart had not been previously described. To determine the activity of AMPKK in the heart, we developed the following method. AMPKK activity was determined by measuring the activation of α_{312}. Kinase buffer A contained 50 mM Tris (pH 8), 80 mM NaCl, 1 mM EDTA, 0.02% (w/v) Brij-35, 1 mM DTT, 100 μM ATP, 10 mM $MgCl_2$, a cocktail of serine/threonine phosphatase inhibitors (P 2850; Sigma), a tyrosine phosphatase inhibitor cocktail (P 5726; Sigma), a protease inhibitor cocktail (P 8340; Sigma) and 5 μg α_{312}, unless otherwise indicated. Reactions were initiated by the addition of sample (3 μg) that was incubated in kinase buffer A with or without α_{312}, for 30 min or for the times indicated. The reactions were then adjusted to contain 50 mM Tris (pH 8), 80 mM NaCl, 1 mM EDTA, 0.02% (w/v) Brij-35, 1 mM DTT, 200 μM ATP, 7.5 mM $MgCl_2$, 1 μCi [γ-^{32}P]-ATP, a serine/threonine phosphatase inhibitor cocktail (P 2850; Sigma), a tyrosine phosphatase inhibitor cocktail (P 5726; Sigma), a

protease inhibitor cocktail (P 8340; Sigma) and 200 μM AMARA peptide. The reactions were further incubated for 10 min at 30°C and subsequently terminated by the addition of H_3PO_4 (1% v/v, final concentration). An aliquot of each reaction was spotted into a well of a Unifilter P81 (Whatman) 96-well filterplate. Each well was washed 10 times with 300 μL of 1% (v/v) H_3PO_4 and twice with methanol. The filterplate was air-dried and 50 μL of MicroScint PS scintillant (Perkin Elmer) was added to each well. Each well of the filterplate was counted in a MicroBeta Trilux (Wallac) scintillation counter. The activity of AMPKK is expressed as the net increase in the activity of α_{312}; 1 U equals 1 pmol of phosphate incorporated into the AMARA peptide per minute.

AMPKK activity was also directly determined by measuring the phosphorylation of Thr 172 within α_{312}. Samples were incubated in kinase buffer A for 60 min and the reactions were terminated by the addition of SDS-PAGE loading buffer. Reactions were subsequently boiled, separated by SDS-PAGE and then transferred to PVDF membranes. Membranes were then immunoblotted for phosphoThr 172, as described above.

2.2.8 *Immunoprecipitations*

The ability of the goat anti-LKB1 antibody (M-18; Santa Cruz Biotechnology) to immunoprecipitate LKB1 was tested by incubating 0, 150, or

300 μg of cleared homogenate with 1 μg of antibody, which was prebound to the wells of a protein A/G coated 8-well strip (Pierce). The strips were incubated with gentle shaking for 4 h at 4°C. Wells were then washed 4 times with a buffer containing 50 mM Tris-HCl (pH 8 at 4°C), 1 mM EDTA, 10% (w/v) glycerol, 0.02% (v/v) Brij-35, 150 mM NaCl, protease inhibitors (P 8340; Sigma), and phosphatase inhibitors (P 2850 and P 5726; Sigma). After the last wash, the immunoprecipitate and supernatant were resuspended in protein sample buffer lacking β-mercaptoethanol. Samples were boiled, resolved by SDS-PAGE, and transferred to PVDF membranes. Membranes were probed first with a rabbit anti-LKB1 antibody (Cell Signaling Technologies) and were then probed with a horseradish peroxidase-coupled donkey anti-rabbit IgG antibody that was preabsorbed to goat IgG (Jackson ImmunoResearch). Immunocomplexes were detected as described above.

All subsequent immunoprecipitations were conducted as described above, except LKB1 was immunoprecipitated from 0 or 150 μg of cleared homogenate and 1 mM DTT was included in the wash buffer. In addition, after the last wash the immunoprecipitate was immediately assayed for AMPKK activity by the aforementioned methods.

2.2.9 *Statistical Analysis*

Differences between the mean values of two groups were determined using an unpaired Student's t-test. $P<0.05$ was considered significant.

2.3 Results

2.3.1 *Cardiac Function and High-Energy Phosphates in Severe Ischemic Hearts*

Hearts were perfused either for 60 min aerobically or for 30 min aerobically followed by 30 min of severe ischemia. As expected, cardiac work ceased in hearts subjected to severe ischemia (62 ± 4 and 0 mm Hg·mL·min^{-1}·10^{-2}, in aerobic (n=10) and ischemic hearts (n=12), respectively). In addition to a depression in cardiac function, 30 min of severe ischemia caused a perturbation in the cellular levels of high energy phosphates that was reflected in an increase in the ratios of AMP:ATP and Cr: PCr (Table 2.1).

2.3.2 *AMPK Activity and ACC Phosphorylation Following Severe Ischemia*

Accordant with previous findings [6, 11], AMPK activity was markedly stimulated in severe ischemic hearts compared to aerobic hearts (Figure 2.1A). During severe ischemia it is not possible to measure the rate of fatty acid oxidation as a downstream marker of AMPK activity; thus, we determined whether the increased activity of AMPK was associated with an increase in ACC

phosphorylation. As expected, a significant increase in the phosphorylation of ACC at Ser 79 was observed in severe ischemic hearts as compared to aerobic hearts (Figure 2.1B). The total abundance of ACC protein was similar between aerobic (0.80 ± 0.03 A.U., n=10) and severe ischemic hearts (0.89 ± 0.05 A.U., n=10).

2.3.3 *Phosphorylation State of AMPK Thr 172 and AMPKK Activity Following Severe Ischemia*

An important mechanism that governs the catalytic activitiy of AMPK is the phosphorylation of the key residue, Thr 172 [23]. Accordingly, the stimulation of AMPK activity following no-flow ischemia was paralleled by an increase in the phosphorylation state of Thr 172 (Figure 2.2A). The total amount of AMPK did not differ between aerobic (1.20 ± 0.05, n=10) and severe ischemic hearts (1.14 ± 0.04 A.U., n=12).

To further investigate the mechanism responsible for the increased phosphorylation state of Thr 172 within AMPK, we determined the effect of severe ischemia on the activity of AMPKK. To accomplish this, we developed an *in vitro* assay that measures the activation of purified recombinant α_{312} by AMPKK. This assay facilitated the measurement of AMPKK activity, which increased in a time-dependent manner when assayed with various

concentrations of the substrate, α_{312} (Figure 2.2B). When AMPKK activity was determined in aerobic and severe ischemic hearts, it was found that ischemia stimulated the activity of AMPKK by 1.8 fold (Figure 2.2C). Importantly, these data provide the first evidence that ischemia stimulates myocardial AMPKK and demonstrates that in the heart, AMPKK is coordinately regulated with AMPK.

2.3.4 *Cardiac Function and Levels of High Energy Phosphates Following Mild Ischemia*

Thus far, AMPK activation has primarily been studied in models of severe myocardial ischemia where dramatic changes in the AMP:ATP and Cr:PCr ratios occur [8]. We attempted to distinguish between the regulatory roles of allosterism and phosphorylation by studying the regulation of AMPK in a model of mild ischemia. A series of hearts was subjected to either 60 min of aerobic perfusion or 30 min of aerobic perfusion followed by 30 min of mild ischemia. In this model of ischemia, coronary flow was reduced by 39% compared to aerobic hearts (23 ± 2 and 14 ± 0.4 $mL \cdot min^{-1}$ in aerobic (n=8) and mild ischemic hearts (n=9), respectively). The reduction in coronary flow resulted in a 53% decrease in cardiac work compared to aerobic hearts (73 ± 8 and 34 ± 4 $mmHg \cdot mL \cdot min^{-1} \cdot 10^{-2}$ in aerobic (n=8) and mild ischemic hearts (n=9), respectively). The decreases in cardiac work and coronary flow remained constant throughout the entire 30 min

of ischemia (data not shown). Despite the impairments in coronary flow and cardiac work, the ratios of AMP:ATP and Cr:PCr were similar between aerobic and mildly ischemic hearts (Table 2.2).

2.3.5 *Effect of Mild Ischemia on AMPK Activity, ACC Phosphorylation and Metabolism*

Although the ratios of AMP:ATP and Cr:PCr were similar between aerobic and mildly ischemic hearts, AMPK activity was significantly elevated in hearts subjected to mild ischemia, as compared to aerobically-perfused hearts (Figure 2.3A). While the activity of AMPK was elevated in mildly ischemic hearts, this did not translate to an increase in the phosphorylation of ACC at Ser 79 (Figure 2.3B). However, a significant increase in glycolytic rates was evident in hearts subjected to mild ischemia (Figure 2.3C).

2.3.6 *Alterations in AMPK Phosphorylation and AMPKK Activity Following Mild Ischemia*

Since the activation of AMPK following mild ischemia occurred in the absence of detectable changes in the ratios of AMP:ATP or Cr:PCr, we addressed whether the activation of AMPK was due to an increase in the phosphorylation state of Thr 172 and to an increase in AMPKK activity. Immunoblot analysis

revealed that while the total abundance of AMPK was similar between aerobic (0.95 ± 0.05 A.U., n=6) and mildly ischemic hearts (0.92 ± 0.02 A.U., n=8), the phosphorylation state of Thr 172 was significantly increased in hearts exposed to mild ischemia (Figure 2.4A). In parallel, the activity of AMPKK was also elevated in hearts subjected to mild ischemia when compared to hearts perfused aerobically (Figure 2.4B). Thus, mild ischemia stimulates AMPKK independently of changes in the AMP:ATP or Cr:PCr ratios. Increased AMPKK activity is the primary mechanism by which mild ischemia stimulates AMPK.

2.3.7 *Effect of Ischemia on LKB1 Protein Abundance and Activity*

Since previous studies have implicated LKB1 as an important AMPKK [26-28] and it has been demonstrated that endurance training, which stimulates AMPK activity [39, 40], results in increased LKB1 protein abundance in skeletal muscle [41], we examined whether LKB1 protein abundance and/or activity is enhanced by ischemia. As depicted in Figure 2.5, LKB1 protein abundance was similar between aerobic and severe ischemic hearts. LKB1 was efficiently immunoprecipitated (Figure 2.6A) and consistent with the results of others [26-28], LKB1 phosphorylated α_{312} at Thr 172 and also activated α_{312} (Figure 2.6B and 2.5C, respectively). Interestingly, the activity of LKB1 was not different between aerobic and severe ischemic hearts (Figure 2.6C) and thus did not correlate with

the activity of AMPKK (Figure 2.2C). Hence an additional AMPKK, other than LKB1, is stimulated by ischemia and regulates AMPK in the heart.

2.4 Discussion

While it is evident that myocardial AMPK activity, Thr 172 phosphorylation, and the ratios of AMP:ATP and Cr:PCr are increased during severe ischemia [6-8, 10], the role of AMPKK in modifying AMPK activity during ischemia has not been previously addressed. Importantly, the present study demonstrates that during severe ischemia, AMPKK activity is stimulated in conjunction with an increase in AMPK activity, Thr 172 phosphorylation, and changes in cellular high energy phosphate content. Additionally, utilizing a model of mild ischemia, we demonstrate an important regulatory role of AMPKK on AMPK activity. Perfusion of hearts under mild ischemic conditions, where coronary flow was reduced by 39%, resulted in decreased cardiac work and increased rates of glycolysis. While such changes typically occur during myocardial ischemia, levels of high energy phosphates may or may not be altered, depending on the severity of ischemia [42], In the model employed in this study, the ratios of AMP:ATP and Cr:PCr were unchanged following mild ischemia, yet a marked enhancement of AMPK activity and Thr 172 phosphorylation was evident. Furthermore, the activation of AMPK by mild

ischemia was accompanied by an increase in AMPKK activity. Our data suggest that mild ischemia stimulates AMPKK, and subsequently AMPK, through a high energy phosphate-independent mechanism.

Although the classical model for activation of the AMPK "cascade" is centered on the role of the positive allosteric effects of the ratios of AMP:ATP [43] and Cr:PCr [19], a study by Beauloye C. *et al.* [8] established precedence for an AMP-independent regulation of AMPK activity in the heart. In other cell types, it has also been shown that AMPK is regulated by various stimuli, which do not alter the AMP:ATP ratio [20, 21, 44]. Our data provide additional evidence that increases in the ratios of AMP:ATP and Cr:PCr are not essential for the activation of myocardial AMPK and AMPKK during ischemia. Rather, we find that myocardial AMPK is primarily regulated via phosphorylation of Thr 172 by AMPKK.

Interestingly, AMPK was activated to a greater degree following severe ischemia than mild ischemia. Despite the differential activation of AMPK, AMPKK was stimulated to a similar extent (1.8 fold). Although it is possible that AMPKK is further stimulated by positive allosterism during severe ischemia, this effect may not have been detected by the *in vitro* assay for AMPKK. Alternatively, positive allosterism may further enhance the activity of phosphorylated AMPK during severe ischemia.

Elevated ratios of AMP:ATP and Cr:PCr may also be necessary to facilitate AMPK-mediated phosphorylation of ACC. Although AMPK was activated with mild and severe ischemia, ACC Ser 79 phosphorylation was increased only with severe ischemia. Alternatively, it is possible that a threshold stimulation of AMPK must be achieved to confer signaling to ACC or that other protein kinases participate in ACC phosphorylation.

Altered subcellular localization of AMPK may also positively affect the activity of AMPK and/or confer signaling specificity. Specifically, while the β subunit was initially shown to function as a scaffold that binds the α and γ subunits [45], recent studies suggest the β subunit additionally regulates the localization of AMPK [46-48]. The two isoforms of the β-subunit contain a glycogen-binding domain that, as its name implies, facilitates its binding to glycogen [47, 48]. Intriguingly, the catalytic activity of AMPK is not affected by glycogen binding [48]. Thus, differences in the degree of AMPK activation and ACC phosphorylation found between severe ischemia and mild ischemia may be explained by an increase in the proportion of AMPK that is not sequestered by glycogen. Although glycogen turnover was not assessed in this study, rates of glycogenolysis have been shown to differ with ischemic severity [49].

Recently, the Ser/Thr protein kinase LKB1 was identified as an AMPKK that is not coordinately stimulated with AMPK [28]. LKB1 was found to co-

purify with liver AMPKK and phosphorylate recombinant AMPK complexes [26, 28]. Strikingly, the activity of LKB1 was not increased by stimuli that increased AMPK activity [28, 30, 31]. In the present study, we also find that LKB1 is not activated with AMPK. Although, immunoprecipitated LKB1 phosphorylated and activated recombinant α_{312}; the activity of myocardial LKB1 was not increased by ischemia. Moreover, LKB1 protein abundance was not altered by ischemia. Taken together, these data demonstrate that the activities of myocardial LKB1 and AMPKK are differentially regulated by ischemia. This suggests that an additional AMPKK, distinct from LKB1 is present in the heart. Unlike LKB1, this AMPKK is coordinately stimulated with AMPK by ischemia. The existence of multiple mammalian AMPKKs is not without precedence, since three AMPKK orthologues have been identified in *S. cerevisiae* [27, 29]. Since the AMPKK characterized in this study plays an important role in the regulation of AMPK and hence metabolism, both during and following ischemia, we are currently determining the identity of this kinase (Chapter 5).

In summary, the present study demonstrates that increased AMPKK activity is the primary mechanism responsible for the stimulation of AMPK during ischemia. In addition, we reveal that global changes in high energy phosphate homeostasis are not necessary for the activation of AMPKK and thus AMPK, but these changes may additionally activate phosphorylated AMPK.

While the results of this study confirm that LKB1 phosphorylates and activates AMPK, we demonstrate that ischemia stimulates an alternate AMPKK, which activates AMPK and thereby mediates changes in metabolism that contribute to cardiac dysfunction.

Table 2.1 Severe Ischemia Increases the AMP:ATP and Cr:PCr Ratios

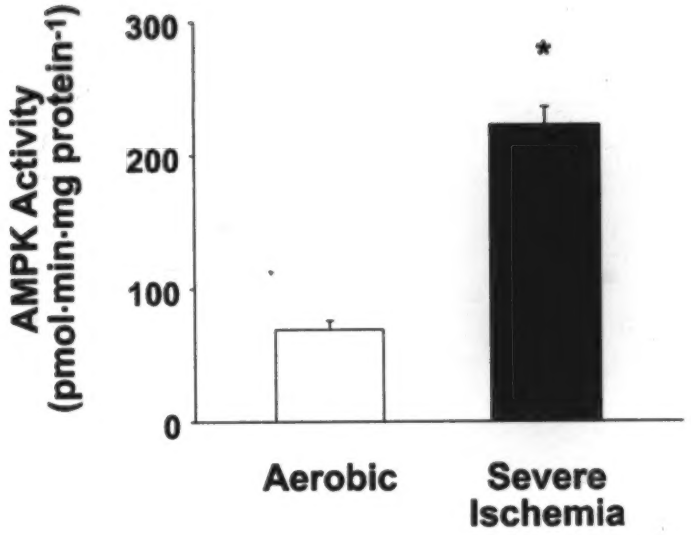
High energy phosphates were measured at the end of 60 min of aerobic perfusion (n=6) or at the end of 30 min of severe ischemia (n=6). Values represent mean ± SEM. * denotes $P<0.05$.

	Aerobic	Severe Ischemia
High Energy Phosphate Metabolism		
AMP:ATP	0.34 ± 0.15	2.32 ± 0.53 *
Cr:PCr	4.47 ± 0.65	10.51 ± 0.79 *

Figure 2.1 Severe Ischemia Stimulates AMPK Signaling

A) AMPK activity in aerobic (n=8) and severe ischemic rat hearts (n=9). B) Densitometric analysis of the phosphorylation state of Ser 79 within ACC in aerobic (n=10) and severe ischemic rat hearts (n=10). Representative immunoblots are shown. Values represent the mean ± SEM. * denotes $P<0.05$.

A.


AMPK Activity
(pmol·min·mg protein-1)
300
200
100
0
*
Aerobic
Severe
Ischemia

B.

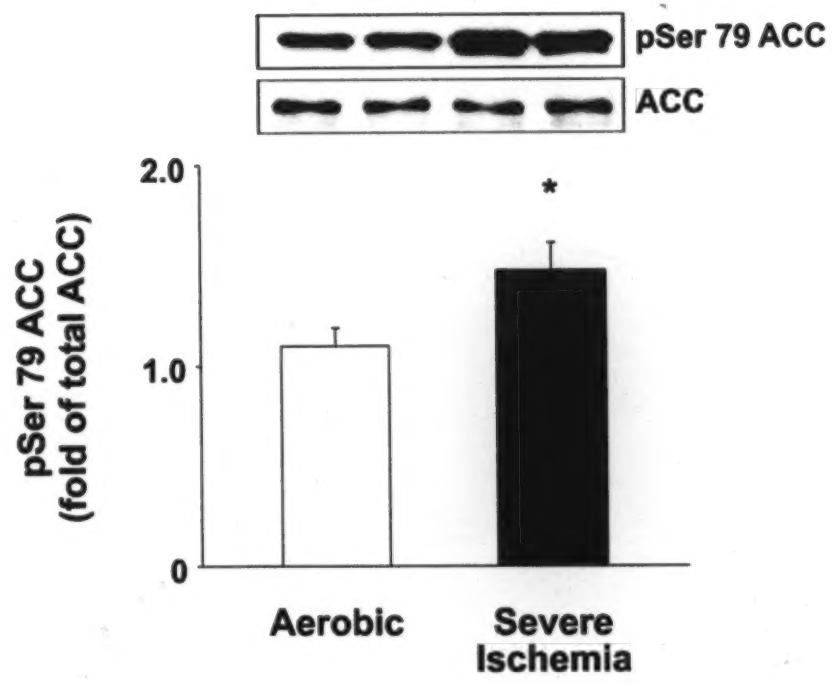
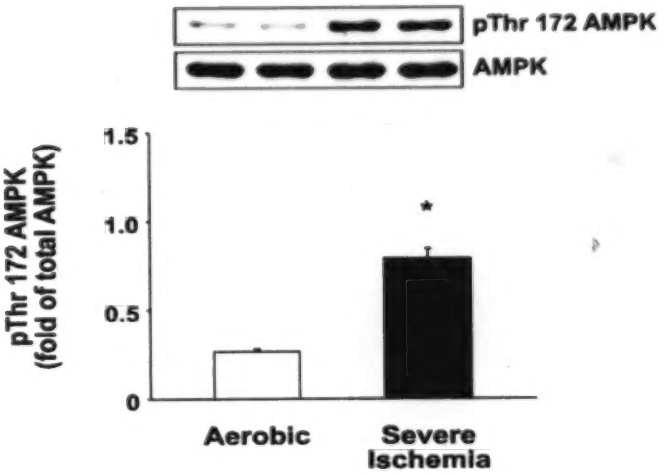

pSer 79 ACC
ACC
pSer 79 ACC
(fold of total ACC)
2.0
1.0
0
*
Aerobic
Severe
Ischemia

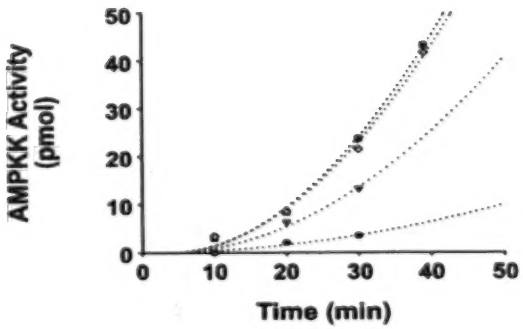
Figure 2.2 Severe Ischemia Stimulates Phosphorylation of AMPK via AMPKK

A) Densitometric analysis of the phosphorylation state of Thr 172 within AMPK in aerobic (n=10) and severe ischemic rat hearts (n=12). Representative immunoblots are shown. B) AMPKK assay progress curve. The activity of AMPKK was determined by measuring the activation of α_{312}. 3 μg of 5% PEG6000 supernatant was incubated for 10, 20, 30 or 39 minutes with the following amounts of α_{312}: 1 μg (filled circles), 2 μg (open triangles), 5 μg (filled squares), or 10 μg (open diamonds). C) AMPKK activity in aerobic (n=9) and severe ischemic rat hearts (n=9). Values represent the mean ± SEM. * denotes $P<0.05$.

A.


pThr 172 AMPK
AMPK
pThr 172 AMPK (fold of total AMPK)
1.5
1.0
0.5
0
*
Aerobic
Severe Ischemia

B.


AMPKK Activity (pmol)
50
40
30
20
10
0
0
10
20
30
40
50
Time (min)

C.

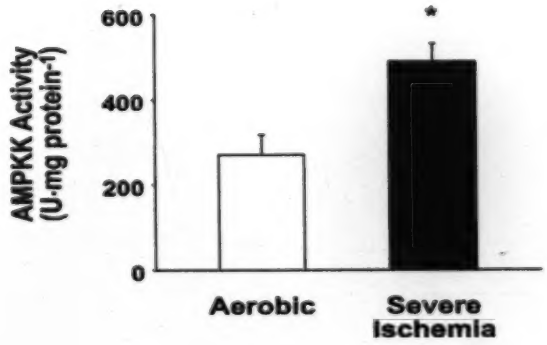

AMPKK Activity (U-mg protein-1)
600
400
200
0
*
Aerobic
Severe Ischemia

Table 2.2 Mild Ischemia does not Affect the Ratios of AMP:ATP and Cr:PCr

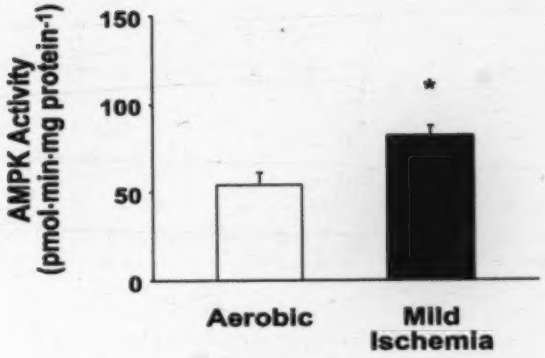
High energy phosphates were measured at the end of 30 min of aerobic perfusion (n=8) or at the end of the 30 min mild ischemic period (n=10). Values represent mean ± SEM.

	Aerobic	Mild Ischemia
High Energy Phosphate Metabolism		
AMP:ATP	0.15 ± 0.05	0.13 ± 0.01
Cr:PCr	2.23 ± 0.42	1.98 ± 0.25

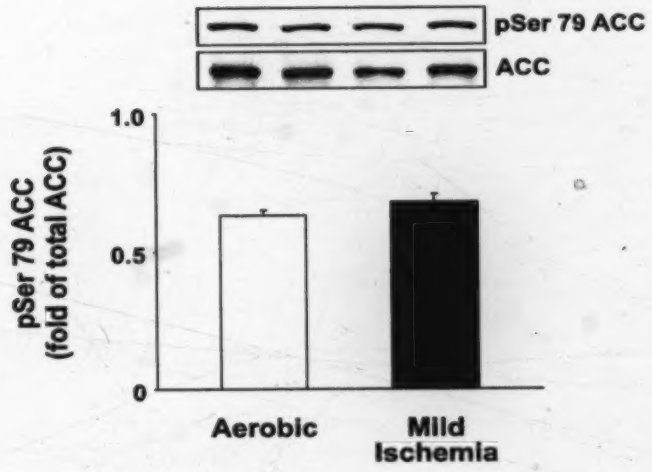
Figure 2.3 Mild Ischemia Stimulates AMPK Signaling with Differential Effects on ACC Phosphorylation and Glycolysis

A) AMPK activity in aerobic (n=6) and mild ischemic rat hearts (n=9). B) Densitometric analysis of the phosphorylation state of Ser 79 within ACC in aerobic (n=6) and mild ischemic rat hearts (n=8). Representative immunoblots are shown. C) Rates of glycolysis in aerobic (n=7) and mild ischemic rat hearts (n=10). Values represent the mean ± SEM. * denotes $P<0.05$.

A.


AMPK Activity
(pmol·min·mg protein-1)
150
100
50
0
*
Aerobic
Mild
Ischemia

B.


pSer 79 ACC
ACC
pSer 79 ACC
(fold of total ACC)
1.0
0.5
0
Aerobic
Mild
Ischemia

C.

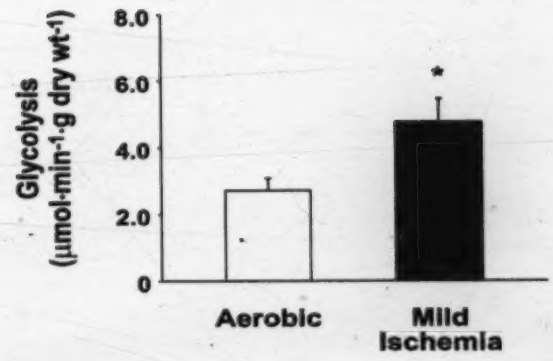
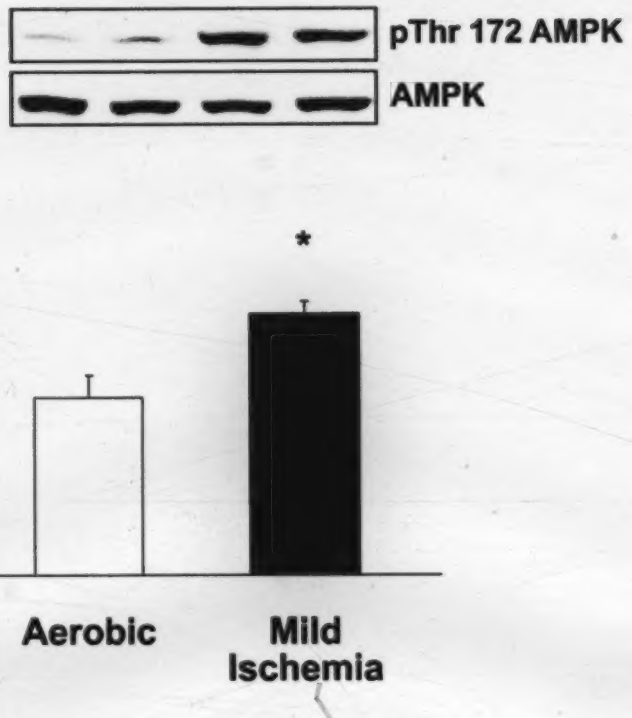

Glycolysis
(μmol·min-1·g dry wt-1)
8.0
6.0
4.0
2.0
0
*
Aerobic
Mild
Ischemia

Figure 2.4 Mild Ischemia Stimulates Phosphorylation of AMPK via AMPKK

A) Densitometric analysis of the phosphorylation state of Thr 172 within AMPK in aerobic (n=6) and mild ischemic rat hearts (n=8). Representative immunoblots are shown. B) AMPKK activity in aerobic (n=7) and mild ischemic rat hearts (n=8). Values represent the mean ± SEM. * denotes $P<0.05$.

A.


pThr 172 AMPK
AMPK
pThr 172 AMPK
(fold of total AMPK)
1.5
1.0
0.5
0
*
Aerobic
Mild
Ischemia

B.

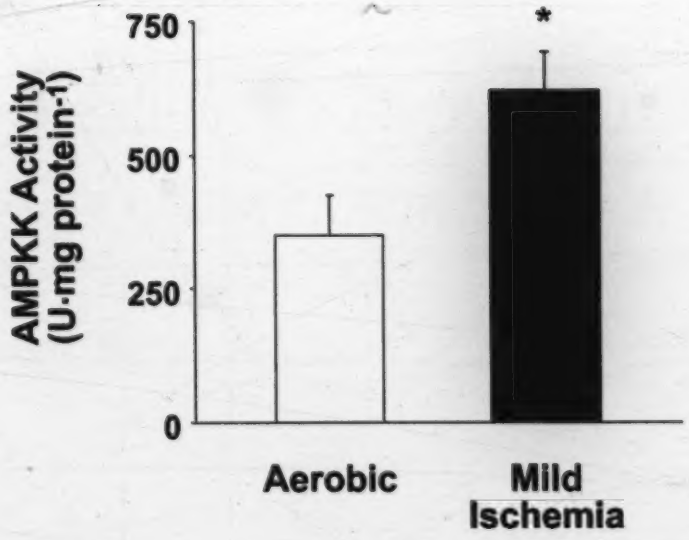

AMPKK Activity
(U·mg protein-1)
750
500
250
0
*
Aerobic
Mild
Ischemia

Figure 2.5 LKB1 Protein Levels are not Altered by Severe Ischemia

Representative immunoblot and densitometric analysis of LKB1 protein levels in aerobic (n=8) and severe ischemic rat hearts (n=10). Values represent the mean ± SEM.

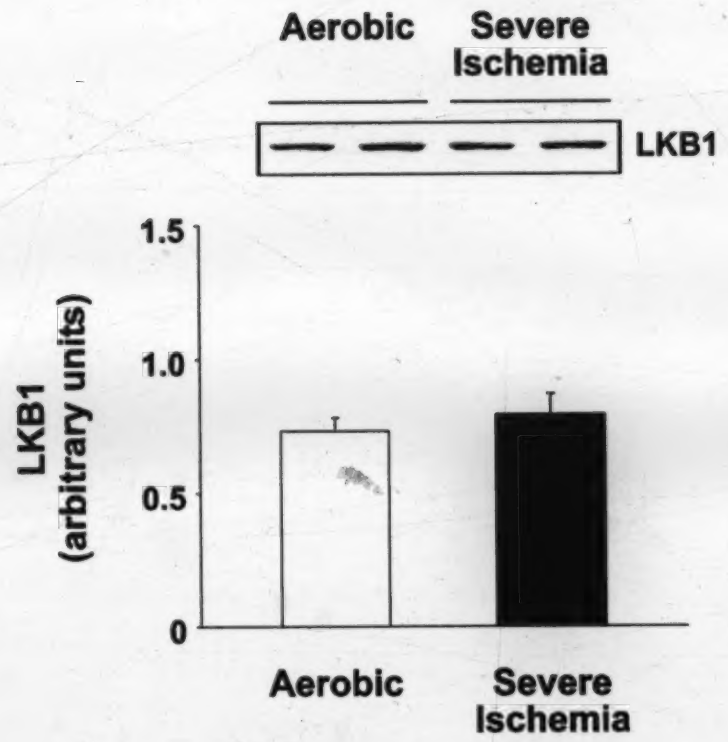
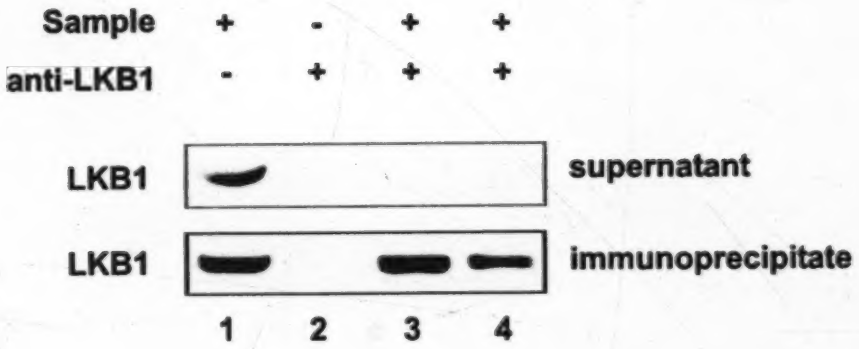

Aerobic
Severe Ischemia
LKB1
LKB1 (arbitrary units)
1.5
1.0
0.5
0
Aerobic
Severe Ischemia

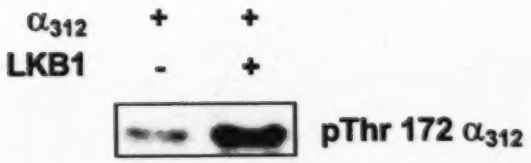
Figure 2.6 LKB1 is not the Myocardial AMPKK Activated by Ischemia

A) LKB1 immunoblot of supernatants and precipitates following immunoprecipitation of LKB1. Lane 1 is a non-immunoprecipitated sample. Lanes 2, 3 and 4 are immunoprecipitates and supernatants obtained from 0, 300 and 150 μg of homogenate, respectively. B) Immunoblot for phosphoThr 172 AMPK following a kinase assay of immunoprecipitated LKB1. Immunoprecipitated LKB1 was incubated with recombinant α_{312} for 60 min. C) Kinase assay of immunoprecipitated LKB1 from aerobic (n=5) and severe ischemic rat hearts (n=5). Immunoprecipitated LKB1 was incubated with α_{312} for 60 min prior to determining the activity of α_{312} as described in Materials and Methods. Values represent the mean ± SEM.

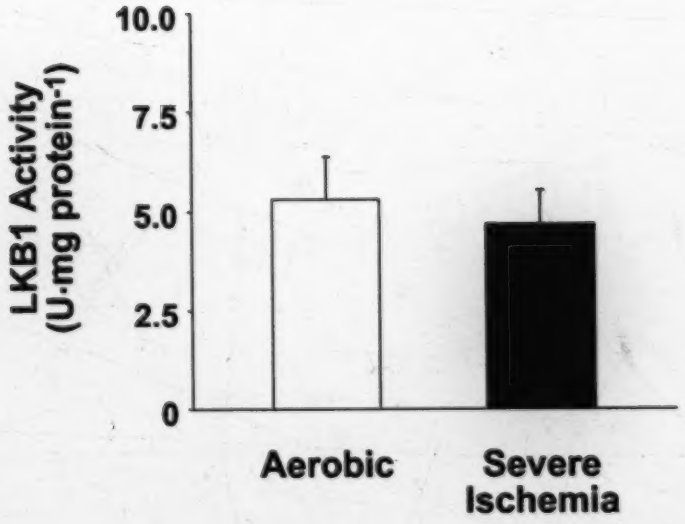
A.


Sample + - + +
anti-LKB1 - + + +
LKB1
supernatant
LKB1
immunoprecipitate
1 2 3 4

B.


α312 + +
LKB1 - +
pThr 172 α312

C.


LKB1 Activity
(U·mg protein-1)
10.0
7.5
5.0
2.5
0
Aerobic
Severe
Ischemia

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Chapter 3

INSULIN PROMOTES GLYCOLYSIS AND GLUCOSE OXIDATION BUT DOES NOT INHIBIT FATTY ACID OXIDATION OR AMPK SIGNALING IN THE ISCHEMIC HEART

3.1 Introduction

The cardioprotective effects of glucose-insulin-potassium (GIK) were first described in the 1960s [1, 2] and have since been supported by additional clinical [3, 4] and experimental studies [4]. A recent meta-analysis of clinical trials conducted in the pre-thrombolytic era, demonstrates that GIK infusion reduces in-hospital mortality after acute myocardial infarction (MI) [5]. In addition, a pilot clinical study recently found that co-administration of high-dose GIK with reperfusion therapy reduces mortality following MI [6]. Moreover, GIK improves left ventricular function and reduces infarct size in experimental ischemia-reperfusion [7, 8]. Although it has been known for several decades that GIK treatment benefits the post-ischemic myocardium, the mechanism of action of GIK has not been fully elucidated.

Currently, three mechanisms, which center on the actions of insulin, have been hypothesized to mediate the salutary effects of GIK on the ischemic myocardium. First, it has been proposed that insulin induces a shift from the oxidation of fatty acids to glucose [9]. Insulin has been found to decrease circulating free fatty acid levels and fatty acid extraction by the human myocardium, when administered systemically [10, 11]. Secondly, with respect to glucose metabolism, insulin directly stimulates glucose uptake, glycogen synthesis, glycolysis and glucose oxidation in the heart [12]. A third hypothesis

suggests that the anti-apoptotic actions of insulin may account for the cardioprotective effects of GIK [9, 13]. Insulin treatment improves contractile function and decreases infarct size in reperfused-ischemic hearts [14-16]. Furthermore, the beneficial effects of insulin correlate with phosphorylation of BAD, which is associated with cell survival [15]. Intriguingly, the Akt pathway is an integral component of the protective mechanism, since pharmacological inhibitors of either upstream or downstream mediators of Akt signaling abrogate the cardioprotective effects of insulin [15, 16].

Although it has been proposed that the metabolic and pro-survival effects of insulin independently confer cardioprotection [13, 15], increasing evidence suggests that they are not mutually exclusive. It is now evident that Akt is not only central to insulin signaling [17], but also mediates crosstalk with AMPK [18-20], a Ser/Thr protein kinase that positively regulates fatty acid oxidation and glucose metabolism [21]. In an early study, insulin was found to inhibit AMPK activity and thereby reduce palmitate oxidation in normoxic hearts perfused with 0.4 mM palmitate [22]. Insulin also inhibits AMPK activity, in hearts perfused in the absence of fatty acids, by decreasing the phosphorylation of an activating site, Thr 172 [18, 19]. Subsequently, it was demonstrated that constitutively active Akt mimics the action of insulin on AMPK Thr 172

phosphorylation [19], suggesting that insulin negatively regulates AMPK via Akt.

Importantly, insulin also negatively regulates AMPK activity and Thr 172 phosphorylation of AMPK during ischemia [18]. As demonstrated in Chapter 2, ischemia stimulates AMPK activity and Thr 172 phosphorylation by activating AMPKK. Thus in the present study, we investigated whether the reciprocal mechanism also holds true; i.e. does insulin inhibit fatty acid oxidation by suppressing AMPKK activity and consequently, AMPK signaling in the ischemic heart?

Recently, Clark *et al.* demonstrated that 0.5 mM palmitate[1] antagonizes the inhibition of AMPK by insulin [23]. In rats and humans, fasting plasma fatty acid levels[1] range from 0.7 to 1.0 mM [24, 25] and 0.3 to 0.6 mM [26, 27], respectively. However, in humans circulating fatty acid levels are further elevated (1.0 to 2.2 mM) in clinically relevant forms of myocardial ischemia [28-31]. Thus, we investigated the effects of insulin on myocardial fatty acid oxidation and AMPK signaling in hearts perfused with high levels of fatty acids (1.2 mM palmitate). Resolution of the mechanistic details underlying the cardioprotective effects of insulin on the ischemic myocardium will greatly facilitate the design of novel therapeutic agents.

[1] The majority of circulating fatty acids are bound to albumin *in vivo* and in *in vitro* studies; unbound fatty acids represent less than 0.005 % of the total amount of fatty acids [32, 33]

3.2 Materials and Methods

3.2.1 *Heart Perfusions*

All of the animals used in this study were cared for in accordance with the guidelines provided by the Canadian Council on Animal Care.

Hearts from fed male Sprague-Dawley rats (300 to 350 g) were isolated and perfused as working heart preparations, as described previously [34, 35]. Hearts were subjected to either 60 min of aerobic perfusion or 30 min of aerobic perfusion followed by 30 min of mild ischemia. The one-way ball valve technique [36] was modified to produce a controlled sustained mild-ischemia, as described in Chapter 2 (2.2.1). Hearts were perfused with a modified Krebs-Henseleit solution that contained 2.5 mM Ca^{2+}, 5.5 mM glucose, 1.2 mM palmitate bound to 3% fatty acid free bovine serum albumin (Cohn fraction V, Sigma), and either 0 or 100 μU/mL insulin.

At the end of the perfusions, all hearts were quickly frozen with Wollenberger clamps cooled to the temperature of liquid N_2. Frozen hearts were ground to a powder using a mortar and pestle cooled to the temperature of liquid N_2. Portions of the frozen powdered tissue were utilized to determine the dry to wet ratio and for biochemical measurements.

As described previously [37], rates of glycolysis, glucose oxidation, and palmitate oxidation were determined by quantitatively measuring the ^3H_2O or

$^{14}CO_2$ produced from hearts perfused with buffer containing D-[5-^{3}H(N)]-glucose (Amersham Biosciences), D-[U-^{14}C]-glucose (Amersham Biosciences), or [9, 10-^{3}H] palmitate (NEN), respectively.

3.2.2 *Protein Extractions*

Protein extractions were conducted as described in Chapter 2.

3.2.3 *Immunoblotting*

Immunoblotting was conducted as described in Chapter 2, except the following primary antibodies, obtained from Cell Signaling Technologies, were utilized: anti-AMPK, anti-phosphoThr 172 AMPK, anti-Akt, anti-phosphoSer 473 Akt, or anti-phosphoSer 79 ACC. Immunoblots for ACC were conducted using horseradish peroxidase-labeled streptavidin (Kirkegaard and Perry Laboratories).

3.2.4 *AMPKK Assay*

AMPKK activity was determined by measuring the activation of α_{312}, as described in Chapter 2.

3.2.5 *Statistical Analysis*

Differences between the mean values were determined using a two way analysis of variance, followed by a Holm-Sidak post-hoc test; $P<0.05$ was considered statistically significant.

3.3 Results

3.3.1 *Effects of Insulin on Cardiac Function*

Isolated working rat hearts were perfused with clinically relevant concentrations of glucose and fatty acids in the presence or absence of insulin. Hearts were subjected to either a 60 min aerobic perfusion or a 30 min aerobic perfusion followed by 30 min of mild ischemia. In aerobically perfused hearts, insulin had no effect on cardiac function (Table 3.1). Likewise, insulin did not affect the mechanical function of hearts subjected to ischemia (Table 3.1).

3.3.2 *Effects of Insulin on Myocardial Glucose and Palmitate Metabolism*

In aerobically perfused hearts, rates of glycolysis (Figure 3.1A) and glucose oxidation (Figure 3.1B) were increased in insulin-treated hearts as compared with control hearts. Similarly, in ischemic hearts, insulin stimulated both glycolysis (Figure 3.1A) and glucose oxidation (Figure 3.1B) as compared with control hearts. Importantly, insulin treatment did not modify the metabolic

response to ischemia. In control hearts, ischemia induced a 1.5-fold increase in glycolysis (Figure 3.1A) and a 1.4-fold increase in glucose oxidation (Figure 3.1B), when compared to aerobic values. Similarly, ischemia stimulated glycolysis by 1.5-fold (Figure 3.1A) and glucose oxidation by 1.3-fold (Figure 3.1B), in insulin-treated hearts. In contrast to glucose metabolism, insulin did not alter rates of palmitate oxidation during aerobic or ischemic perfusion (Figure 3.1C). In addition, rates of palmitate oxidation were similar during the aerobic and the ischemic periods, in control and insulin-perfused hearts (Figure 3.1C). Although insulin stimulated a marginal increase in the calculated rates of ATP production in aerobic (93 and 105 μmol ATP·g dry·min^{-1}, control and insulin-treated hearts, respectively) and ischemic hearts (94 and 96 μmol ATP·g dry·min^{-1}, control and insulin-treated hearts, respectively), the values are within the margin of error. In summary, insulin promotes the metabolism of glucose but not the oxidation of fatty acids in aerobic and ischemic hearts.

3.3.3 *Role of Insulin in the Regulation of AMPK*

Consistent with the signaling actions of insulin, Akt phosphorylation at Ser 473 (Figure 3.2A) was stimulated in aerobic hearts treated with insulin. In contrast to previous studies performed in hearts perfused with no or low levels of fatty acids [18, 19], the phosphorylation state of AMPK at Thr 172 was not

inhibited in insulin-treated aerobic hearts (Figure 3.2B). Further examination of the AMPK signaling pathway revealed that neither the activity of the upstream kinase, AMPKK (Figure 3.3A), nor the phosphorylation of the downstream target of AMPK, ACC (Figure 3.3B), was affected by insulin. Thus, insulin does not regulate AMPKK and hence AMPK signaling in the aerobic heart perfused with high levels of fatty acids.

Although Beauloye *et al.* reported that the inhibition of AMPK by insulin is more pronounced in ischemic hearts perfused in the absence of fatty acids [18], this was not evident in this study, where high levels of fatty acids were utilized. Similar to aerobic hearts, insulin stimulated Akt phosphorylation (Figure 3.2A) in ischemic hearts. However, AMPK Thr 172 phosphorylation (Figure 3.2B), AMPKK activity (Figure 3.3A) and ACC Ser 79 phosphorylation (Figure 3.3B) were unchanged between control and insulin-treated ischemic hearts. These data do not support earlier suggestions that insulin and/or Akt inhibits AMPKK and AMPK in the heart [18, 19].

3.4 Discussion

In the current study, we demonstrate that insulin does not directly regulate palmitate oxidation in the aerobic or ischemic heart, perfused with high levels of fatty acids. In accordance with these findings we clearly demonstrate

that insulin does not regulate the AMPK signaling pathway, in the aerobic or ischemic heart perfused with high levels of fatty acids, as the activity of AMPKK, the phosphorylation of AMPK, and the phosphorylation of ACC are unchanged following insulin treatment. Additionally, we demonstrate that insulin primarily regulates glucose metabolism and specifically, stimulates both glycolysis and glucose oxidation in aerobic and ischemic hearts.

It has been hypothesized that insulin stimulates a shift in myocardial metabolism from fatty acid oxidation to glucose oxidation, and thereby improves post-ischemic cardiac function [9]. The present study affirms that insulin directly promotes exogenous glucose metabolism in aerobic and ischemic hearts perfused with high levels of fatty acids. However, our data reveal that exogenous palmitate oxidation is not concomitantly inhibited by insulin, under aerobic or ischemic conditions. Under normoxic conditions, previous studies have shown that systemic administration of insulin inhibits myocardial fatty acid extraction [10, 11], whereas intracoronary infusion of insulin does not [10]. Thus, circulating free fatty acid levels may be an important determinant of insulin's effects on myocardial fatty acid metabolism.

In accordance, insulin has recently been found to inhibit myocardial fatty acid oxidation in hearts perfused with 0.5 or 0.4 mM palmitate [22, 38, 39], but not when hearts were perfused with 1.2 mM palmitate [40]. Importantly, in the

aforementioned studies similar concentrations of glucose (10 or 11 mM) were employed [22, 38-40]. However in the latter study, Sakamoto *et al.* also established that insulin-mediated inhibition of fatty acid oxidation does not occur in hearts perfused with lower concentrations of glucose and palmitate (5 mM and 0.4 mM, respectively) [40]. Similarly, Atkinson *et al.* have demonstrated that palmitate oxidation is not inhibited by insulin when hearts are perfused with 5 mM glucose and 0.8 mM palmitate [41]. In the present study, hearts were perfused with 5.5 mM glucose and 1.2 mM palmitate to mimic the plasma glucose and free fatty acid levels seen in the clinical setting of myocardial ischemia [28, 42]. Thus, our data support the notion that elevated levels of fatty acids and/or lower glucose concentrations serve to abrogate insulin-mediated inhibition of palmitate oxidation.

The evident dichotomy in the regulation of fatty acid oxidation by insulin is also apparent with the regulation of AMPK. Insulin has been reported to inhibit the activity and/or phosphorylation of AMPK in some studies [18, 19, 22] but not in others [40, 41]. In the present study, inhibition of AMPK phosphorylation was not evident in aerobic or ischemic hearts. Furthermore, key upstream and downstream components of the AMPK pathway were also unchanged following insulin treatment; no changes in the activity of AMPKK,

the phosphorylation of ACC, or fatty acid oxidation were evident following insulin treatment, in aerobic or ischemic hearts.

In addition to exogenous supplies of glucose and fatty acids, endogenous stores of glycogen and triglycerides serve as important fuels for the heart [37, 43, 44]. Since the effects of insulin on glycogen and triglyceride metabolism were not examined, we cannot exclude the possibility that insulin stimulation of exogenous glucose metabolism is accompanied by an inhibition of glycogen and/or triglyceride turnover. Altered glycogen and/or triglyceride metabolism would balance the marginal increase in the calculated rate of total ATP production (from exogenous sources) observed with insulin treatment. Moreover, it would be of interest to examine whether insulin-mediated changes in triglyceride metabolism are associated with altered levels of malonyl-CoA, an inhibitor of mitochondrial fatty acid uptake [45].

Of note, this study also demonstrates that ischemia selectively inhibits insulin signaling to Akt but does not impair insulin-stimulated glucose metabolism. In aerobic and ischemic hearts, rates of glycolysis and glucose oxidation were increased to the same extent. However, while insulin increased Akt phosphorylation by 2.9-fold in aerobic hearts, insulin caused a 1.9-fold increase in Akt phosphorylation in ischemic hearts. These findings corroborate previous reports, which independently showed that the ischemic heart is insulin-

sensitive with respect to glucose uptake [46], but exhibits blunted insulin signaling through Akt [47]. Our data further suggests that ischemia selectively abrogates insulin-Akt signaling and does not impair insulin's ability to stimulate myocardial glucose metabolism.

In the model of mild ischemia utilized in this study, insulin did not improve cardiac function. However, any beneficial effects of insulin attributed to a lowering of circulating fatty acid levels would not be present in the model utilized in this study.

In summary, we demonstrate that insulin promotes glucose metabolism but does not regulate palmitate oxidation or AMPK signaling in the aerobic and ischemic heart. Additionally, we find that ischemia selectively impairs insulin signaling through Akt but does not inhibit the regulation of glucose metabolism by insulin.

Table 3.1 Insulin does not Affect Mechanical Function of Aerobic and Ischemic Rat Hearts

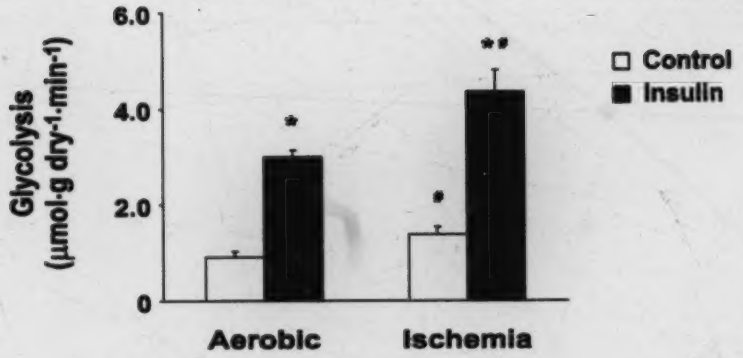
Heart rate, peak systolic pressure, cardiac output, coronary flow and cardiac work in isolated working rat hearts perfused with or without insulin (100 μU/mL). Hearts were subjected to a 60 min aerobic perfusion or a 30 min aerobic perfusion followed by 30 min of mild ischemia. Values are the mean ± SEM of 15 to 17 hearts measured at the end of the perfusion period. * denotes $P<0.05$ versus aerobic hearts within the respective control or insulin-treated group.

	AEROBIC		ISCHEMIC	
	Control	Insulin	Control	Insulin
Heart Rate (beats·min^{-1})	254 ± 4	262 ± 3	230 ± 6 #	225 ± 8 #
Peak Systolic Pressure (mm Hg)	129 ± 3	127 ± 2	121 ± 5	117 ± 6
Cardiac Output (mL·min^{-1})	62 ± 3	60 ± 2	26 ± 2 #	24 ± 2 #
Coronary Flow (mL·min^{-1})	20 ± 0	19 ± 1	17 ± 1 #	16 ± 1 #
Cardiac Work (mL·mm Hg·min^{-1}·10^{-2})	80 ± 4	76 ± 3	32 ± 3 #	29 ± 3 #

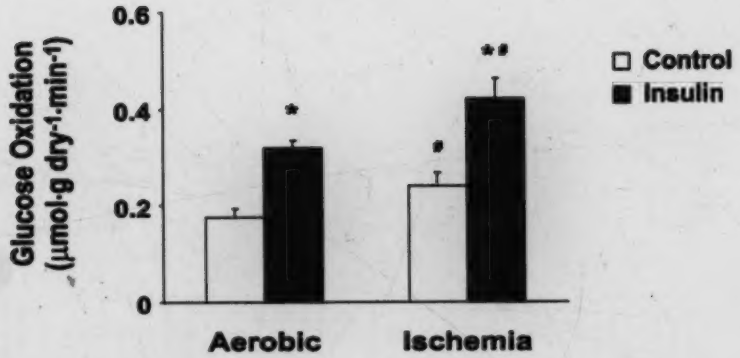
Figure 3.1 Insulin Stimulates Glucose Metabolism but does not Alter Palmitate Oxidation in Aerobic and Ischemic Rat Hearts

A) Rates of glycolysis in aerobic hearts (n=10, control; n=10, insulin) and ischemic hearts (n=8, control; n=9, insulin). B) Rates of glucose oxidation in aerobic hearts (n=7, control; n=7, insulin) and ischemic hearts (n=7, control; n=8, insulin). C) Rates of palmitate oxidation in aerobic hearts (n=10, control; n=10, insulin) and ischemic hearts (n=8, control; n=9, insulin). Values are the mean ± SEM measured between 20 and 60 min of aerobic perfusion or between the 10 and 30 min of the ischemic period. * denotes a difference between insulin-treated hearts and control hearts, within the respective aerobic or ischemic perfusion condition. ' denotes a difference between ischemic and aerobic hearts, within the respective control or insulin-treated group.

A.


Glycolysis
(μmol·g dry⁻¹·min⁻¹)
6.0
4.0
2.0
0
*
**
#
Aerobic
Ischemia
Control
Insulin

B.


Glucose Oxidation
(μmol·g dry⁻¹·min⁻¹)
0.6
0.4
0.2
0
*
**
#
Aerobic
Ischemia
Control
Insulin

C.

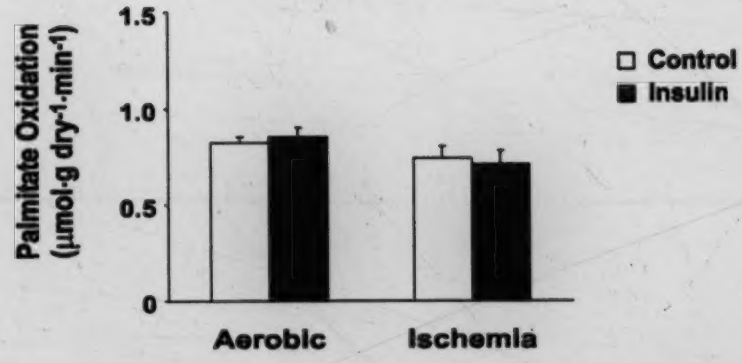
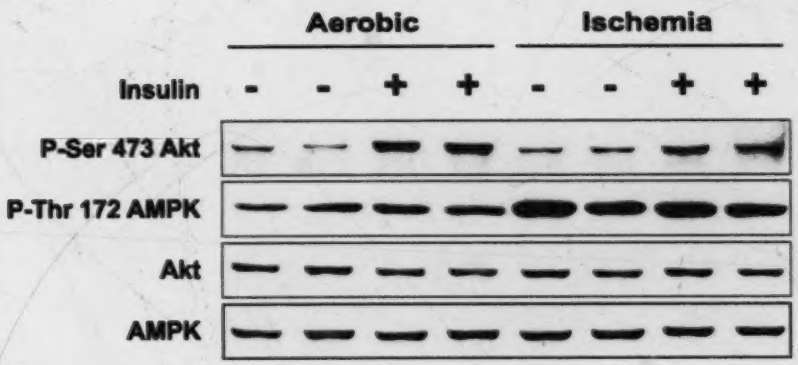

Palmitate Oxidation
(μmol·g dry⁻¹·min⁻¹)
1.5
1.0
0.5
0
Aerobic
Ischemia
Control
Insulin

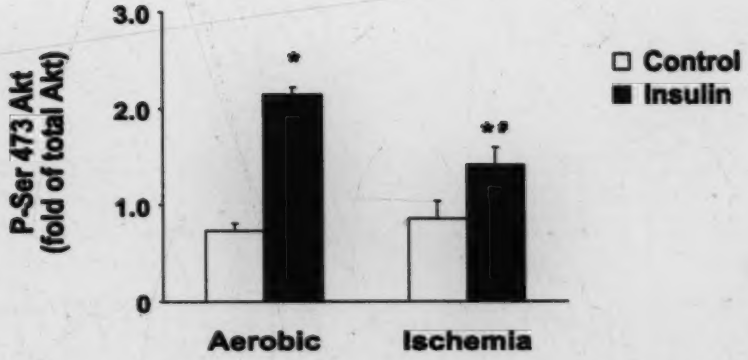
Figure 3.2 Insulin Increases Phosphorylation of Akt, but not AMPK

A) Representative immunoblots, B) densitometric analysis of Akt Ser 473 phosphorylation and C) densitometric analysis of AMPK Thr 172 phosphorylation in aerobic (n=7, control; n=7, insulin) and ischemic rat hearts (n=9, control; n=8, insulin). Values are the mean ± SEM. * denotes a difference between insulin-treated hearts and control hearts, within the respective aerobic or ischemic perfusion condition. ' denotes a difference between ischemic and aerobic hearts, within the respective control or insulin-treated group.

A.


Aerobic
Ischemia
Insulin - - + + - - + +
P-Ser 473 Akt
P-Thr 172 AMPK
Akt
AMPK

B.


P-Ser 473 Akt
(fold of total Akt)
3.0
2.0
1.0
0
*
*#
Aerobic
Ischemia
Control
Insulin

C.

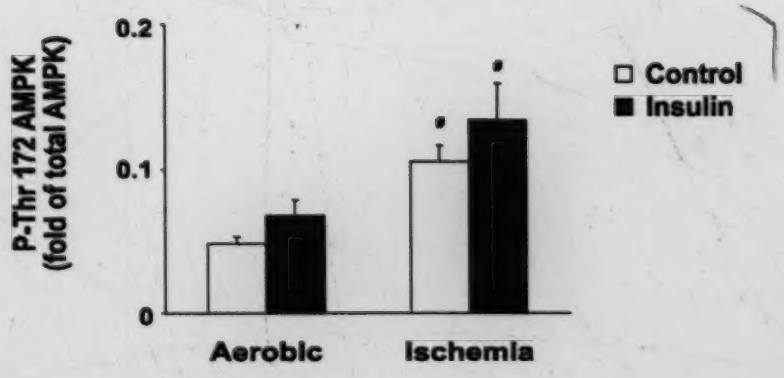
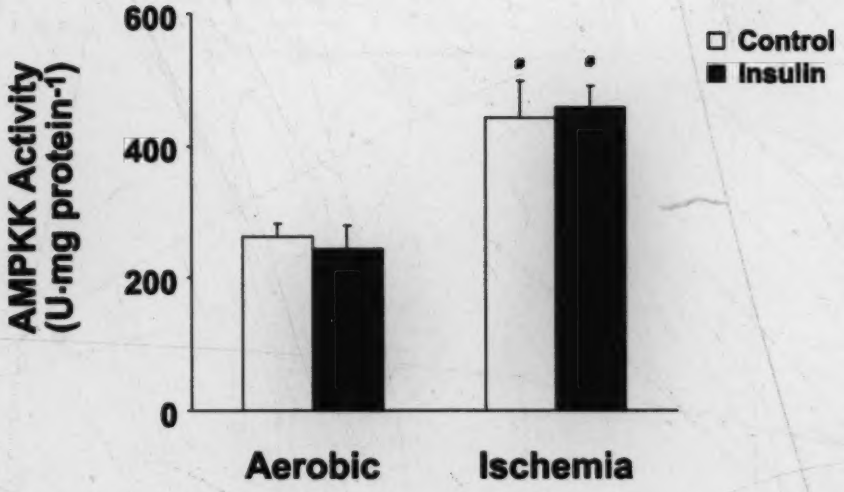

P-Thr 172 AMPK
(fold of total AMPK)
0.2
0.1
0
#
#
Aerobic
Ischemia
Control
Insulin

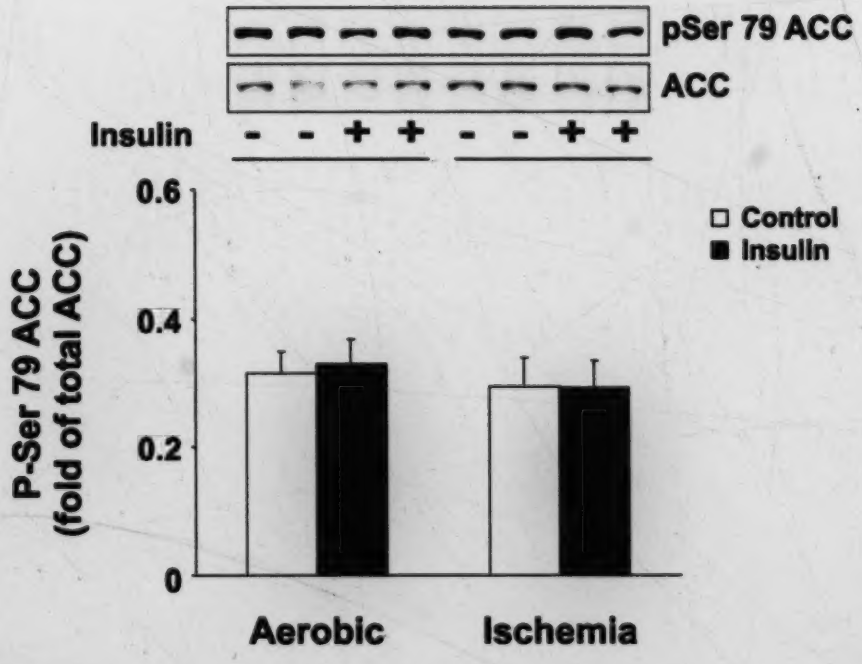
Figure 3.3 Insulin does not Inhibit AMPKK Activity or ACC Phosphorylation in Aerobic and Ischemic Rat Hearts

A) Effect of insulin on AMPKK activity in aerobic (n=8, control; n=7, insulin) and ischemic hearts (n=8, control; n=8, insulin). B) Representative immunoblots and densitometric analysis of ACC Ser 79 phosphorylation in aerobic (n=7, control; n=7, insulin) and ischemic hearts (n=9, control; n=8, insulin). Values are the mean ± SEM. ' denotes a difference between ischemic and aerobic hearts, within the respective control or insulin-treated group.

A.


600
400
200
0
AMPKK Activity
(U·mg protein-1)
Control
Insulin
Aerobic
Ischemia

B.


pSer 79 ACC
ACC
Insulin
- - + + - - + +
0.6
0.4
0.2
0
P-Ser 79 ACC
(fold of total ACC)
Control
Insulin
Aerobic
Ischemia

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Chapter 4

Adiponectin and the Globular Domain of Adiponectin Exert Opposite Effects on Cardiac Glucose and Fatty Acid Oxidation

4.1 Introduction

The hormone adiponectin is an abundant plasma protein [1, 2] that is expressed [2-5] and secreted [2] by adipose tissue. Importantly, reduced plasma concentrations of adiponectin in humans are associated with obesity [1], non-insulin dependent diabetes [6, 7], and coronary artery disease [6, 8, 9] in humans. Furthermore, lowered levels of plasma adiponectin correlate with an increased risk of myocardial infarction (MI) independent of glycemic status and body weight [10], and in fact, plasma levels of adiponectin have been found to decrease further after the onset of MI [11].

Experimental studies have further established that decreased adiponectin levels are causally linked to the development of insulin resistance and cardiovascular disease. Adiponectin-deficient mice are more susceptible to diet-induced insulin resistance [12, 13], exhibit impaired endothelium-dependent vasorelaxation [14], have increased neointimal hyperplasia following injury [12], and display impaired angiogenesis following ischemia [15]. Reciprocally, treatment of obese insulin-resistant *db/db* or KKAy mice with adiponectin improves glucose tolerance, and ameliorates both hyperglycemia and hyperinsulinemia [16]. In addition, overexpression of adiponectin in apolipoprotein E-deficient mice reduces the development of atherosclerotic lesions [17].

Stucturally, adiponectin is composed of a N-terminal collagen domain and a C-terminal globular domain [2]. Adiponectin exists in at least three bioactive oligomeric states: trimers, hexamers, and higher molecular weight (HMW) complexes [2, 18]. Formation of the trimeric adiponectin state is primarily mediated by the C-terminal globular domain [18], while formation of hexameric and HMW complexes of adiponectin additionally requires disulfide-bond formation within the N-terminus [18, 19]. Adiponectin may also be proteolytically processed, as a smaller fragment of adiponectin has been detected in human [13, 20] and mouse plasma [13]. At present it is unknown whether proteolysis of adiponectin *in vivo* generates a bioactive peptide. However, many studies have reported that the globular domain of adiponectin (gAd) is bioactive [18, 20, 21]. Moreover, Pajvani *et al.* recently demonstrated *in vitro*, that trimeric adiponectin is cleaved by a cell-surface protease at amino acid 45, which results in a more potent adiponectin that lacks a portion of the N-terminal collagen domain [19].

The biological actions that mediate adiponectin's metabolic and cardiovascular effects are not completely understood. However, it is evident that adiponectin lowers plasma glucose levels by stimulating glucose uptake in skeletal muscle [21, 22] and by inhibiting hepatic gluconeogenesis [23, 24]. Additionally, adiponectin lowers free fatty acid levels [20] and stimulates fatty

acid oxidation in muscle cells [21]. These metabolic effects of adiponectin are associated with an increase in insulin sensitivity [13, 23, 25] and a stimulation of 5'AMP-activated protein kinase (AMPK) signaling in both liver [21] and skeletal muscle [18, 21, 22]. Interestingly, gAd can potently mimic both the signaling and metabolic effects of adiponectin in skeletal muscle [21]. However, in liver gAd neither activates AMPK [21, 23] nor inhibits hepatic glucose production. The tissue-specific differences between gAd and adiponectin may be attributed, in part, to the differential tissue distribution of adiponectin receptor isoforms [26].

Despite the abundance of data linking adiponectin to obesity [1, 27], non-insulin dependent diabetes [6, 7], coronary artery disease [6, 8, 9], and more recently MI [10, 11], no studies have determined what effect adiponectin has on cardiac function and metabolism in adult hearts. Since, both adiponectin and gAd play important roles in glucose and fatty acid homeostasis [20, 21, 23, 24], we investigated whether adiponectin or gAd elicit metabolic effects on the heart, and if so, whether these effects occur through AMPK and/or insulin signaling. In addition, since alterations in myocardial metabolism have pronounced effects on post-ischemic cardiac function [28, 29], we also examined whether gAd affects the mechanical recovery of reperfused-ischemic hearts.

4.2 Materials and Methods

4.2.1 *Heart Perfusions*

All animal procedures followed the guidelines of the Canadian Council on Animal Care. Isolated hearts from fed male CD1 mice were perfused as working preparations at a 11.5 mm Hg left atrial preload and a 50 mm Hg aortic afterload, as described previously [30]. Hearts were perfused with a modified Krebs-Henseleit solution containing 5.5 mM glucose, 0.4 mM palmitate bound to 3% fatty acid free bovine serum albumin (Sigma), 2.5 mM $CaCl_2$ and 100 μU/mL insulin. After 5 min of aerobic perfusion, phosphate-buffered saline (vehicle), adiponectin or gAd was added. The final concentration of adiponectin or gAd in the perfusate was 1.3 μg/mL. Hearts were perfused aerobically for a total of 40 min. A separate group of hearts was perfused for 10 min and then perfused for an additional 10 min with vehicle, adiponectin (1.3 μg/mL), or gAd (1.3 μg/mL), in the presence or absence of insulin (100 μU/mL). Another series of hearts were subjected to a 30 min aerobic perfusion followed by 18 min of global no-flow ischemia and 40 min of aerobic reperfusion. Vehicle or gAd (1.3 μg/mL) was added to the perfusate after 5 min of aerobic perfusion. At the end of the perfusion period, all hearts were quickly frozen with Wollenberger clamps cooled to the temperature of liquid N_2. Hearts were then powdered under liquid

N_2. Portions of the frozen powdered tissue were utilized to determine the dry to wet ratio and for biochemical measurements.

As described previously [31], rates of glycolysis, glucose oxidation, and palmitate oxidation were determined by quantitatively measuring the ^3H_2O or $^{14}CO_2$ produced from hearts perfused with buffer containing D-[5-^{3}H(N)]-glucose (Amersham Biosciences), D-[U-^{14}C]-glucose (Amersham Biosciences), or [9, 10-^{3}H] palmitate (NEN), respectively.

4.2.2 *Production of Recombinant Adiponectin and gAd*

Recombinant adiponectin and gAd were produced by the method of Fruebis *et al.* [20], except that the murine adiponectin cDNA was cloned into the vector pET30a using the restriction sites *Eco*RV and *Xho*I, which was transformed into the *E. coli* strain BL21(DE3), and adiponectin was purified from the lysed bacterial pellets using Ni^{2+}-immobilized chelating sepharose FF (Amersham Biosciences).

4.2.3 *Separation of Adiponectin Multimers by Velocity Sedimentation*

Recombinant adiponectin and protein standards (Novagen) were separated on a 5 to 20% sucrose gradient, as described previously (28). Gradients were spun in 3.2 mL centrifuge tubes at 259000 *g* for 259 min in a SW55Ti

ultracentrifuge rotor (Beckman). Sequentially, 160 μL fractions were removed and subjected to Western blot analysis with an anti-His_6 horseradish peroxidase labeled antibody (Santa Cruz Biotechnology).

4.2.4 *Protein Extractions*

Heart tissue was homogenized in a buffer containing 50 mM Tris-HCl (pH 8 at 4°C), 1 mM ethylenediamine tetra-acetic acid (EDTA), 10% (w/v) glycerol, 0.02% (v/v) Brij-35, 1 mM dithiothreitol (DTT), protease inhibitors (P 8340; Sigma), and phosphatase inhibitors (P 2850 and P 5726; Sigma). The homogenate was then centrifuged at 13000 *g* for 20 min at 4°C. The protein concentration of the resulting supernatant was determined by the method of Bradford [32].

4.2.5 *AMPK Assay*

AMPK activity was assayed in homogenates as described in Chapter 2.

4.2.6 *Immunoblotting*

Immunoblotting was conducted as described in Chapter 2, except the following primary antibodies, obtained from Cell Signaling Technologies, were utilized: anti-AMPK, anti-phosphoThr 172 AMPK, anti-Akt, anti-phsophoThr308 Akt, or anti-phosphoSer 79 ACC. Blots for ACC content were probed with

horseradish peroxidase-labeled streptavidin (Kirkegaard and Perry Laboratories).

4.2.7 *Statistical Analysis*

Statistical differences between the means of two groups were determined by using a Student's *t*-test. The mean values of more than two groups were compared using a one-way analysis of variance (ANOVA) followed by a Student-Newman-Keuls post-hoc test. A one-way repeated measures ANOVA followed by a Holm-Sidak post-hoc test was used to determine differences within a group over time.

4.3 Results

4.3.1 *Effects of Adiponectin and gAd on Cardiac Function and Metabolism*

To investigate the regulation of myocardial metabolism by adiponectin and gAd, isolated working mouse hearts were perfused with vehicle, adiponectin or gAd for 40 min, and the rates of glycolysis, glucose oxidation and palmitate oxidation were measured. Throughout the perfusion period, cardiac work was stable in hearts treated with vehicle, adiponectin or gAd (Figure 4.1), and neither adiponectin nor gAd altered myocardial contractile function (Table 4.1). Rates of glycolysis were also unaffected by adiponectin or gAd, as

compared with vehicle-treated hearts (Figure 4.2A). However, glucose oxidation rates were stimulated by adiponectin, and inhibited by gAd (Figure 4.2B), when compared to vehicle treatment. In contrast, gAd stimulated palmitate oxidation (Figure 4.2C). Although adiponectin treatment tended to inhibit palmitate oxidation, this effect was not statistically significant (Figure 4.2C).

Importantly, the metabolic effects of adiponectin and gAd did not alter rates of total ATP production (Figure 4.3). Rather, adiponectin and gAd stimulated switches in fuel selection for oxidative metabolism (Figure 4.3). While, the relative contribution of glucose and palmitate oxidation to total ATP production was 43% and 36%, respectively in vehicle treated hearts, the relative contributions to ATP production shifted to 58% and 21%, respectively, for adiponectin-treated hearts (Figure 4.3). In striking contrast, glucose oxidation generated only 28% of the total ATP and palmitate oxidation generated 52% of the total ATP in gAd-treated hearts (Figure 4.3). Thus, adiponectin and gAd reciprocally regulate oxidative metabolism in the heart. These results starkly contrast the parallel stimulation of glucose uptake by adiponectin and gAd that has been observed in skeletal muscle [21].

4.3.2 *Myocardial AMPK Signaling Following Adiponectin and gAd Treatment*

In skeletal muscle, it has been established that both adiponectin and gAd increase fatty acid oxidation by stimulating AMPK [21]. However, unlike skeletal muscle, gAd stimulates myocardial palmitate oxidation whereas adiponectin does not. Thus, we examined whether the opposing effects of adiponectin and gAd on glucose and fatty acid oxidation arise from a differential regulation of AMPK. After 40 min of treatment, no changes in AMPK activity (Figure 4.4A) were observed in hearts perfused with either adiponectin or gAd. Since, it has been reported that AMPK activation in skeletal muscle peaks within minutes of treatment with adiponectin or gAd [21], additional hearts were perfused for 10 min with vehicle, adiponectin or gAd. However, the activity of AMPK was not altered (Figure 4.4B).

4.3.3 *Oligomerization State of Adiponectin*

Recently, it was demonstrated that different oligomeric complexes of adiponectin signal through distinct pathways [18]. Specifically, trimeric adiponectin stimulates AMPK signaling, whereas hexameric and HMW complexes of adiponectin do not [18]. Since, adiponectin did not stimulate AMPK signaling in the heart, we investigated whether this is due to the oligomeric state of adiponectin. Purified recombinant adiponectin and standards

of known molecular weight were subjected to velocity sedimentation through a sucrose gradient. Immunoblot analysis revealed that the purified recombinant adiponectin used in this study predominantly consists of a mixture of HMW and hexameric complexes (Figure 4.5). Since, only small amounts of trimeric adiponectin were detected (Figure 4.5), our data provide further evidence that hexameric and HMW adiponectin complexes do not signal through AMPK.

4.3.4 *Dependence of Adiponectin and gAd Signaling on Insulin*

Since, adiponectin stimulated myocardial glucose oxidation through an AMPK-independent mechanism, we sought to further delineate the participating signaling pathways. In the liver, adiponectin, but not gAd, enhances insulin sensitivity [23]. In endothelial cells, adiponectin stimulates the phosphorylation of Akt [33], a mediator of insulin signaling [34]. Furthermore, in cardiomyocytes, overexpression of constitutively active Akt stimulates glucose metabolism [35]. Therefore, we explored the possibility that adiponectin increases glucose oxidation by stimulating Akt. Immunoblot analysis revealed that 10 min of adiponectin treatment enhanced Akt phosphorylation on Thr 308 as compared with vehicle-treated hearts (Figure 4.6). In contrast, a marked inhibition of Akt phosphorylation was observed in gAd-treated hearts as compared with vehicle-treated hearts (Figure 4.6).

To determine whether adiponectin and gAd modulate Akt phosphorylation in an insulin-dependent manner, we perfused hearts with vehicle, adiponectin or gAd, in the absence of insulin. As expected, the phosphorylation state of Akt Thr 308 was lower in hearts perfused in the absence of insulin (Figure 4.7A) than in insulin-perfused hearts (Figure 4.6). No changes in the phosphorylation state of Akt Thr 308 were evident in hearts that were treated with adiponectin (Figure 4.7A). Furthermore, in the absence of insulin, adiponectin failed to stimulate glucose oxidation (Figure 4.7B). Thus, our data suggests that adiponectin stimulates myocardial glucose oxidation by enhancing insulin signaling to Akt.

Interestingly, in the absence of insulin, gAd did not inhibit Akt phosphorylation (Figure 4.7A) or glucose oxidation (Figure 4.7B). In the absence of insulin, the activity of AMPK (Figure 4.8) was unaffected by gAd treatment when compared to vehicle treatment. Thus, these data indicate that gAd stimulates fatty acid oxidation and inhibits glucose oxidation in the heart, through a mechanism that requires insulin signaling.

4.3.5 *Effect of gAd on Metabolism and Contractile Function in Reperfused-Ischemic Hearts*

Although stimulation of fatty acid oxidation does not perturb contractile function in the normal aerobic heart, the combination of elevated and repressed rates of fatty acid and glucose oxidation, respectively, contribute to the poor contractile recovery of reperfused-ischemic hearts [36]. In addition, activation of Akt improves post-ischemic cardiac function [35], while activation of AMPK is associated with impaired post-ischemic recovery [37, 38]. Since, gAd inhibited Akt, inhibited glucose oxidation, and stimulated fatty acid oxidation, we hypothesized that gAd would exacerbate ischemia-reperfusion injury. To examine this, isolated working mouse hearts were subjected to a 30 minute aerobic perfusion that was followed by 18 min of no-flow ischemia and 40 min of aerobic reperfusion. After 10 min of aerobic reperfusion, the rate pressure product (RPP) in vehicle-treated hearts recovered to 74 ± 9% of pre-ischemic values and remained stable during the remaining 30 min of reperfusion (Figure 4.9A). Similar to vehicle-treated hearts, the rate pressure product in gAd-treated hearts recovered to 73 ± 6% of pre-ischemic values after 10 min of reperfusion (Figure 4.9A). However, the rate pressure product significantly declined to 50 ± 7% of pre-ischemic values by the end of the 40 min of reperfusion (Figure 4.9A). As a result, the functional recovery of gAd-treated hearts was significantly lower

than vehicle-treated hearts during the last 30 min of reperfusion (Figure 4.9A). This depressed post-ischemic recovery of gAd-treated hearts was accompanied by a significant decrease in the total amount of glucose oxidized during reperfusion (Figure 4.9B).

4.4 Discussion

In this study, we demonstrate that adiponectin and gAd elicit diametrically opposed effects on myocardial glucose and fatty acid oxidation rates. In particular, while adiponectin promotes a shift in energy production toward glucose oxidation as opposed to fatty acid oxidation, gAd mediates the converse effect. We also demonstrate that adiponectin enhances insulin signaling through the protein kinase Akt, which may explain the shift towards glucose oxidation. In contrast, the gAd-mediated reduction in glucose oxidation was accompanied by an inhibition of insulin signaling to Akt and a stimulation of palmitate oxidation. Importantly, we also demonstrate that gAd treatment impairs the functional recovery of reperfused-ischemic hearts, and that this detrimental effect is associated with a decrease in glucose oxidation.

In the present study, adiponectin treatment did not alter glycolytic rates but caused a robust stimulation of glucose oxidation in isolated working mouse hearts. Adiponectin treatment resulted in a 15% increase in the contribution of

glucose oxidation to overall ATP production and a 14% decrease in the contribution of palmitate oxidation to ATP production. The observed changes in myocardial metabolism were paralleled by an increase in the phosphorylation of Akt at Thr 308, but were not accompanied by changes in AMPK signaling. The activity of AMPK was not affected by adiponectin treatment. The activation of Akt by adiponectin corroborates the findings of a recent study [33], which found that adiponectin stimulates Akt in endothelial cells. However, dissimilar to skeletal muscle and liver [21], adiponectin does not stimulate palmitate oxidation or AMPK in the heart. In contrast, adiponectin decreased the contribution of fatty acids to ATP production. Since trimeric adiponectin, but not hexameric or HMW adiponectin, stimulates AMPK [18], these discrepancies may be explained by differences in the oligomerization state of adiponectin used. The preparation of adiponectin used in the present study was primarily composed of hexameric and HMW complexes of adiponectin, and contained only small amounts of trimeric adiponectin.

Notably, the adiponectin-dependent changes in the phosphorylation of Akt and glucose oxidation were blunted when insulin was omitted from the perfusate; suggesting that insulin signaling is required for the adiponectin-mediated activation of Akt and stimulation of glucose oxidation. These data provide the first evidence that adiponectin stimulates glucose oxidation and Akt

in the heart, and are consistent with the insulin-sensitizing role of adiponectin, which has been reported in liver [23].

In contrast to adiponectin, gAd stimulates fatty acid oxidation and inhibits glucose oxidation in the heart. Several studies have now established that gAd stimulates skeletal muscle fatty acid oxidation by activating AMPK [21, 22]. However, we did not observe an effect on AMPK in gAd-treated hearts. To determine if AMPK was rapidly and transiently activated, we perfused hearts for a 10 min period with gAd, but also did not observe an activation of AMPK. Moreover, we have previously shown that gAd does not stimulate AMPK in newborn rabbit hearts [39]. Interestingly, gAd also inhibited Akt phosphorylation. Given the stimulatory role of Akt on myocardial glucose metabolism [35, 40], gAd-mediated inhibition of Akt may explain the inhibition of glucose oxidation and the stimulation of fatty acid oxidation. This became evident when hearts were treated with gAd in the absence of insulin. In hearts perfused without insulin no changes in Akt phosphorylation or glucose oxidation were evident. Thus, gAd modifies myocardial oxidative metabolism by inhibiting Akt in an insulin-dependent manner.

The results of this study highlight that the effects of adiponectin and gAd on the heart differ significantly from the effects observed in skeletal muscle [21, 22]. Specifically, while adiponectin and gAd stimulate AMPK in muscle [21, 22],

neither adiponectin nor gAd stimulates AMPK signaling in the heart. In skeletal muscle, both adiponectin and gAd stimulate glucose uptake and fatty acid oxidation [21, 22]. In contrast, adiponectin and gAd exert reciprocal effects on both glucose and fatty acid oxidation in the heart. These differences may be attributed to the varied tissue distribution of the three recently identified adiponectin receptors: AdipoR1 [26], AdipoR2 [26] and T-cadherin [41]. Transcripts for AdipoR1 and AdipoR2 have been detected in the heart by Northern analysis [26]. AdipoR1 binds gAd with high affinity and poorly binds adiponectin [26]. Additionally, gAd binding to AdipoR1 transduces signals, which stimulate AMPK and fatty acid oxidation in C2C12 cells [26]. AdipoR2 binds both adiponectin and gAd with intermediate affinity [26]. Overexpression of AdipoR2 in C2C12 cells enhances the stimulation of fatty acid oxidation by gAd or adiponectin [26]. At present, it is unclear whether AdipoR2 binds trimeric, hexameric or HMW adiponectin. However, since previous studies [18] and the present study demonstrate that hexameric adiponectin does not regulate AMPK and thus fatty acid oxidation, we hypothesize that AdipoR2 binds trimeric adiponectin or trimeric gAd.

Recently, Hug *et al.* identified T-cadherin as a receptor for hexameric and HMW adiponectin by expression cloning [41]. T-cadherin, also named H-cadherin or heart cadherin, belongs to the cadherin superfamily and is

structurally distinct from other family members since it lacks a transmembrane domain and an intracellular domain [42]. Classically, cadherins function in mediating cell-cell interactions [43]. Of note, however, some members of the cadherin superfamily also modulate growth factor receptor signaling by directly interacting with the receptor [44-46]. For example, VE-cadherin promotes vascular endothelial growth factor (VEGF) receptor signaling through Akt in endothelial cells [47], and N-cadherin promotes fibroblast growth factor (FGF) receptor signaling in neurons [44]. Furthermore, the stimulation of FGF receptor signaling by N-cadherin is mediated by extracellular interactions between N-cadherin and the FGF receptor [44]. Thus, it is plausible that T-cadherin may also modify adiponectin receptor signaling. Since, T-cadherin is highly expressed in the cardiovascular system [48] and adiponectin enhances Akt phosphorylation in endothelial cells [33] and the heart, it is possible that adiponectin-receptor signaling to Akt may be enhanced in cell types that express T-cadherin.

Although AMPK signaling to ACC plays an important role in regulating malonyl-CoA levels, and thus fatty acid oxidation in the heart [37, 38], malonyl-CoA metabolism is also governed by malonyl-CoA decarboxylase (MCD) [49, 50]. In the heart, inhibition of MCD results in a stimulation of glucose oxidation

and an inhibition of fatty acid oxidation [51]. Thus, MCD may be a target effector of adiponectin and/or gAd signaling.

In addition to a contributory role in the pathogenesis of insulin resistance [6, 7], reduced plasma adiponectin levels also contribute to the development of coronary artery disease [6, 8, 9]. In addition, recent clinical studies have established that plasma adiponectin levels are inversely correlated with the risk of MI [10] and that after the onset of MI, plasma adiponectin levels further decline [11]. At present, the mechanism responsible for the rapid reduction in adiponectin levels post-MI are unknown, but may include the proteolytic cleavage of adiponectin. The present study further suggests that gAd impairs contractile function in the reperfused-ischemic heart. Given the detrimental effects of gAd on myocardial recovery post-ischemia, further investigation into the mechanisms responsible for the decline in plasma adiponectin levels is warranted.

In conclusion, adiponectin and gAd elicit opposite effects on myocardial metabolism. In the heart, adiponectin stimulates glucose oxidation by enhancing insulin signaling through Akt. In contrast, gAd promotes the preferential oxidation of fatty acids over glucose through the inhibition of insulin signaling through Akt. While the metabolic effects of gAd do not result in altered cardiac function under aerobic conditions, gAd impairs the contractile recovery of

reperfused-ischemic hearts, and this detrimental effect of gAd is associated with an inhibition of glucose oxidation.

Metabolic therapies that stimulate glucose oxidation and/or inhibit fatty acid oxidation are beneficial to the ischemic myocardium [28, 29]. Thus, gAd- and adiponectin-receptor signaling represent potential therapeutic targets in the treatment of myocardial ischemia, as inhibition of gAd signaling or stimulation of adiponectin signaling would favor the use of glucose as an oxidative substrate and thereby enhance cardiac recovery following an ischemic episode.

Figure 4.1 Adiponectin and gAd do not Affect Cardiac Work in Aerobically-Perfused Isolated Working Mouse Hearts

Cardiac work during aerobic perfusion with vehicle (n=23), 1.3 μg/mL adiponectin (Ad) (n=14), or 1.3 μg/mL gAd (n=21). Values are the mean ± SEM.

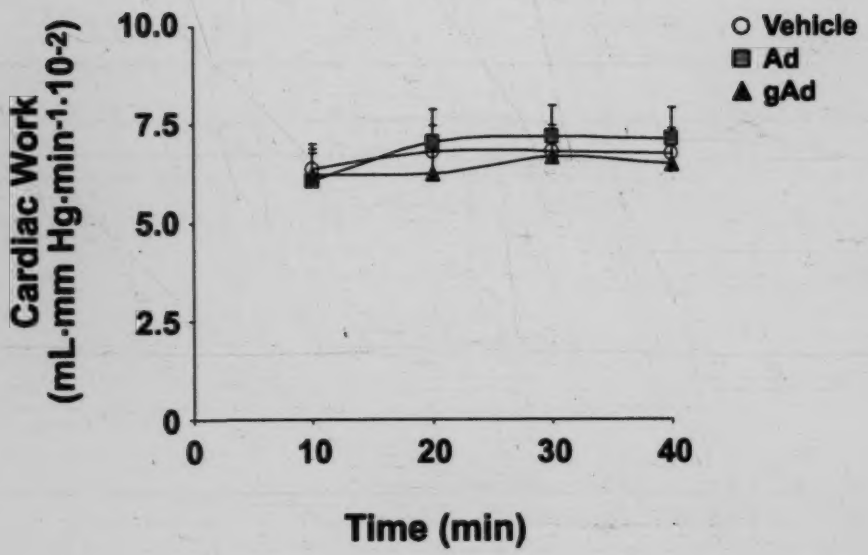

Cardiac Work
(mL·mm Hg·min-1·10-2)
10.0
7.5
5.0
2.5
0
0
10
20
30
40
Time (min)
Vehicle
Ad
gAd

Table 4.1 Adiponectin and gAd do not Affect the Mechanical Function of Aerobically-Perfused Isolated Working Mouse Hearts

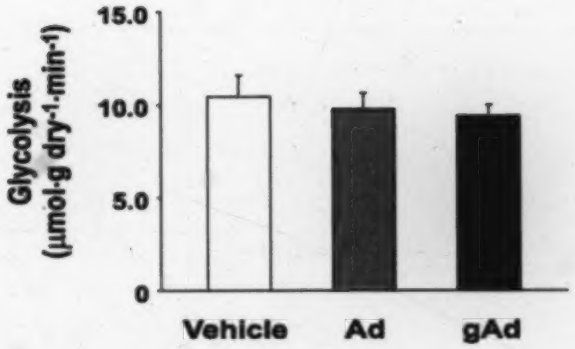
Heart rate, peak systolic pressure, and cardiac output in isolated working mouse hearts after 40 min of aerobic perfusion with vehicle, adiponectin (1.3 μg/mL), or gAd (1.3 μg/mL). Values are the mean ± SEM of 14 to 23 hearts.

	Vehicle	Adiponectin	gAd
Heart Rate ($beats \cdot min^{-1}$)	269 ± 12	284 ± 21	295 ± 13
Peak Systolic Pressure (mm Hg)	70 ± 2	71 ± 3	69 ± 2
Cardiac Output ($mL \cdot min^{-1}$)	9.5 ± 0.8	9.9 ± 0.9	9.2 ± 0.8

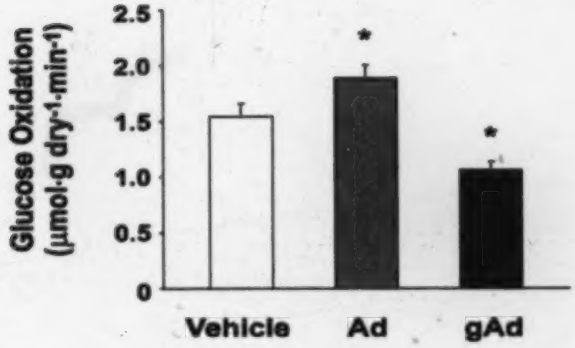
Figure 4.2 Adiponectin and gAd Alter Myocardial Glucose and Fatty Acid Metabolism

Isolated working mouse hearts were perfused for 40 min with vehicle, adiponectin (Ad; 1.3 μg/mL), or gAd (1.3 μg/mL). A) Rates of glycolysis are shown (n=6 vehicle, n=5 Ad, n=5 gAd). B) Rates of glucose oxidation are shown (n=15 vehicle, n=7 Ad, n=9 gAd). C) Rates of palmitate oxidation are shown (n=17 vehicle, n=12 Ad, n=16 gAd). Values are the mean ± SEM. * denotes P<0.05 versus vehicle treatment.

A.


Glycolysis
(µmol·g dry-1·min-1)
15.0
10.0
5.0
0
Vehicle
Ad
gAd

B.


Glucose Oxidation
(µmol·g dry-1·min-1)
2.5
2.0
1.5
1.0
0.5
0
*
*
Vehicle
Ad
gAd

C.

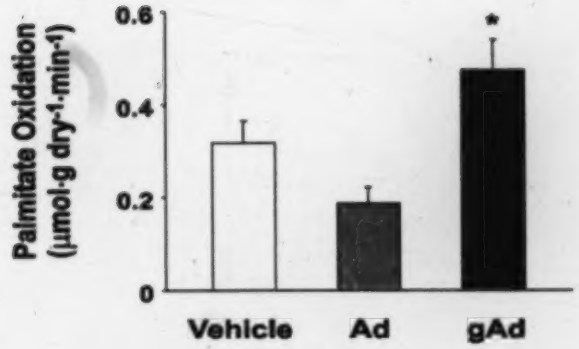

Palmitate Oxidation
(µmol·g dry-1·min-1)
0.6
0.4
0.2
0
*
Vehicle
Ad
gAd

Figure 4.3 Adiponectin and gAd Induce Shifts in the Contribution of Glycolysis, Glucose Oxidation and Palmitate Oxidation to ATP Production

Contribution of glycolysis, glucose oxidation and fatty acid oxidation to the calculated rate of total ATP production in isolated working mouse hearts perfused for 40 min with vehicle, adiponectin (Ad; 1.3 μg/mL), and gAd (1.3 μg/mL). ATP production was calculated by assuming that glycolysis and glucose oxidation produce 2 and 30 mol of ATP per mol of glucose metabolized, respectively, and 105 mol of ATP are produced per mol of palmitate oxidized [52].

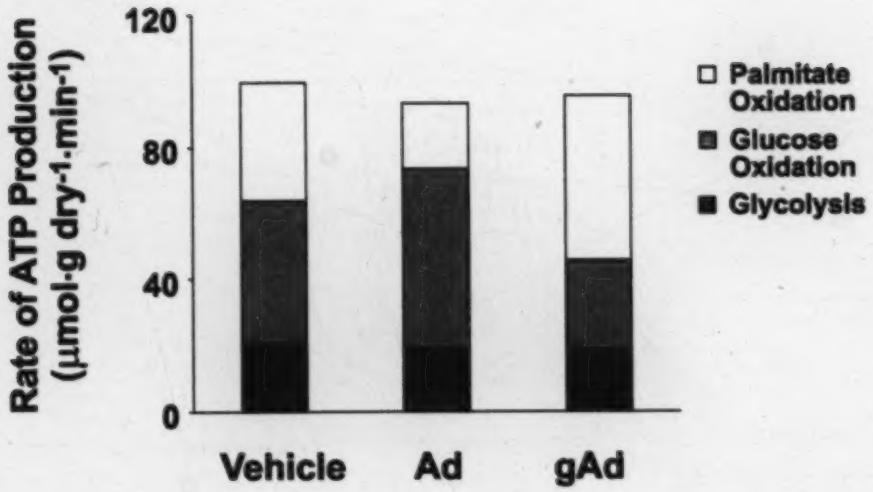
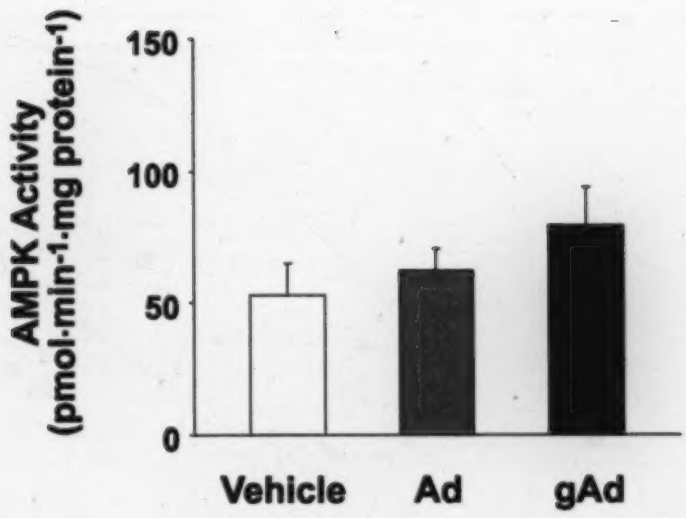

Rate of ATP Production
(μmol-g dry-1-min-1)
120
80
40
0
Vehicle
Ad
gAd
Palmitate Oxidation
Glucose Oxidation
Glycolysis

Figure 4.4 AMPK Activity is not Stimulated in Hearts Treated with Adiponectin or gAd for 40 Min or 10 Min

Activity of AMPK in isolated working mouse hearts at the end of 40 min (A) or 10 min (B) of perfusion with vehicle (n=5), adiponectin (Ad; 1.3 μg/mL; n=7), or gAd (1.3 μg/mL; n=6). Values are the mean ± SEM.

A.


AMPK Activity
(pmol·min-1·mg protein-1)
150
100
50
0
Vehicle
Ad
gAd

B.

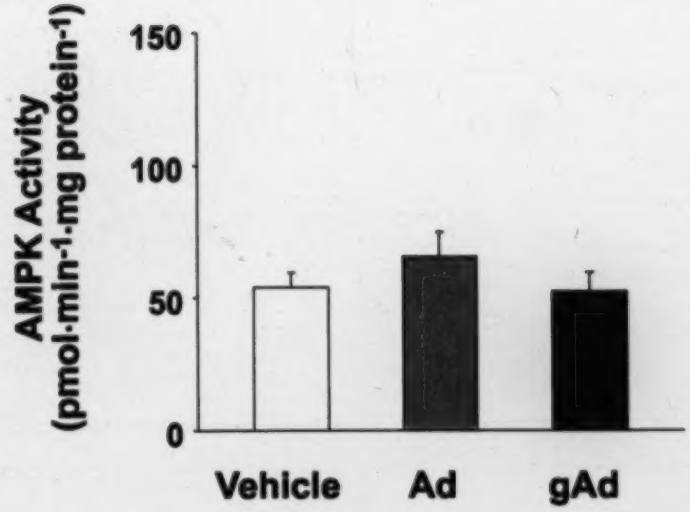

AMPK Activity
(pmol·min-1·mg protein-1)
150
100
50
0
Vehicle
Ad
gAd

Figure 4.5 Oligomerization State of Purified Recombinant Adiponectin

Immunoblot analysis of recombinant adiponectin multimers separated by sucrose-density ultracentrifugation is shown. Solid arrows indicate the position in the gradient where standards of known molecular weights separated into. Dashed arrows denote an extrapolated molecular weight determined from a standard curve generated from the separation of protein standards with known molecular weights.

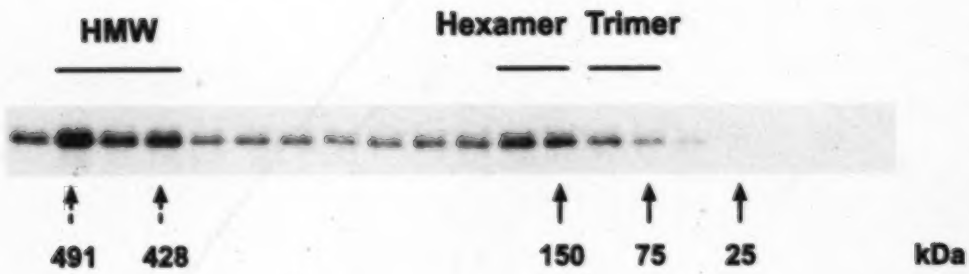

HMW
Hexamer
Trimer
491
428
150
75
25
kDa

Figure 4.6 Akt Phosphorylation is Reciprocally Regulated by Adiponectin and gAd

Representative immunoblots and densitometric analysis of the phosphorylation state of Akt at Thr 308 in isolated working mouse hearts that were perfused for 10 min with vehicle (n=12), adiponectin (Ad; 1.3 μg/mL; n=6), or gAd (1.3 μg/mL; n=7). Values are the mean ± SEM. * denotes P<0.05 versus vehicle treatment.

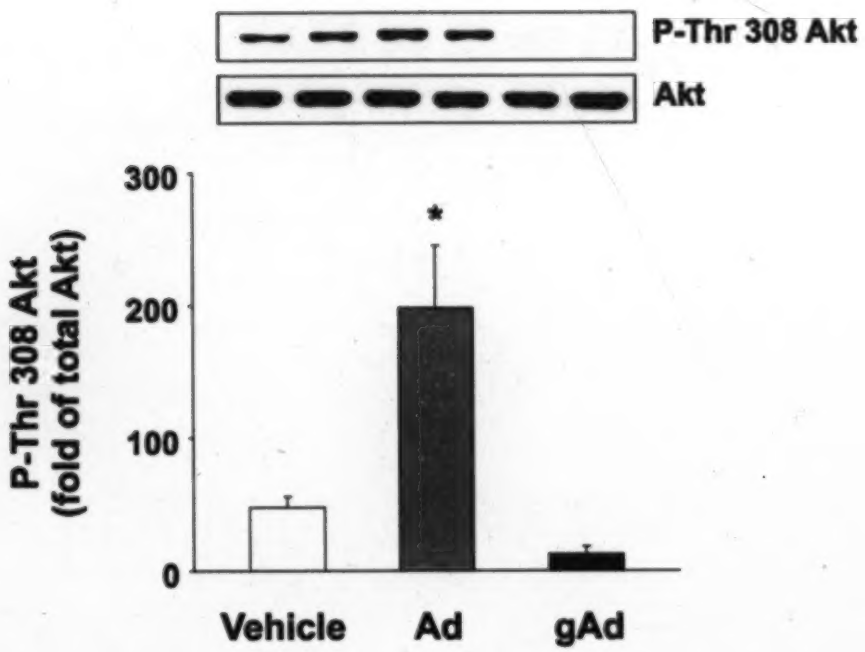
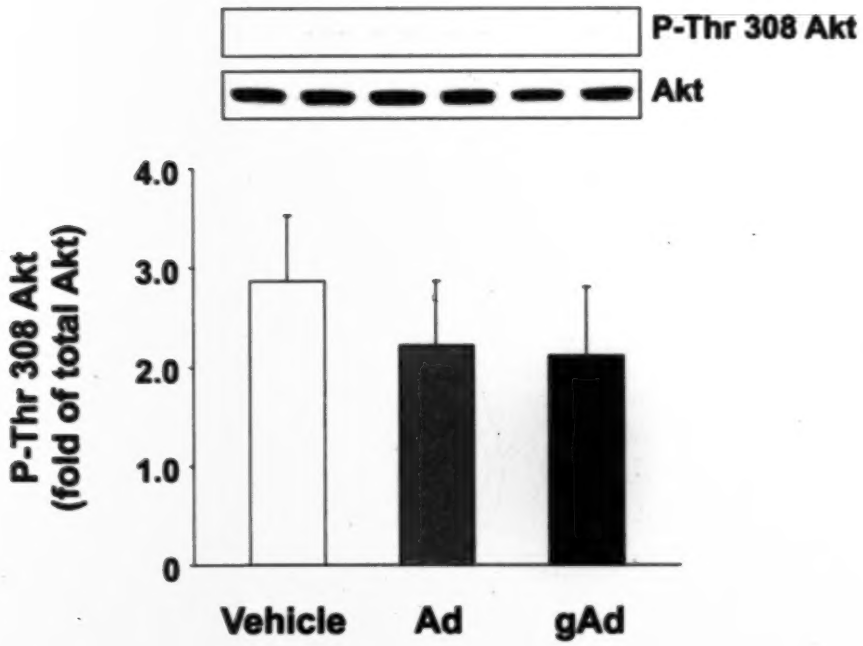

P-Thr 308 Akt
Akt
P-Thr 308 Akt
(fold of total Akt)
300
200
100
0
*
Vehicle
Ad
gAd

Figure 4.7 Adiponectin and gAd do not Affect Akt Phosphorylation and Glucose Oxidation in Isolated Working Mouse Hearts Perfused in the Absence of Insulin

Isolated working mouse hearts were perfused for 10 min without insulin and were treated with vehicle (n=5), adiponectin (Ad; 1.3 μg/mL; n=5), or gAd (1.3 μg/mL; n=4). A) Representative immunoblots and densitometric analysis of the phosphorylation state of Akt at Thr 308 are shown. B) Rates of glucose oxidation are depicted. Values are the mean ± SEM

A.


P-Thr 308 Akt
Akt
P-Thr 308 Akt
(fold of total Akt)
4.0
3.0
2.0
1.0
0
Vehicle
Ad
gAd

B.

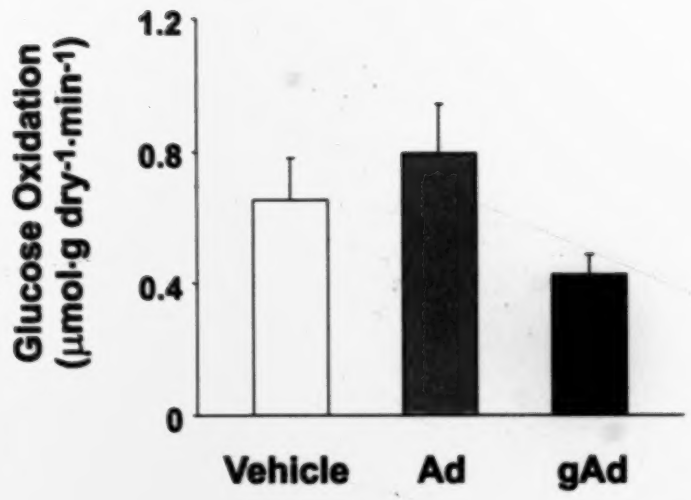

Glucose Oxidation
(μmol·g dry-1·min-1)
1.2
0.8
0.4
0
Vehicle
Ad
gAd

Figure 4.8 Adiponectin and gAd do not Alter AMPK Activity in Isolated Working Mouse Hearts Perfused in the Absence of Insulin

AMPK activity in isolated working mouse hearts were perfused for 10 min without insulin and were treated with vehicle (n=5), adiponectin (Ad; 1.3 μg/mL; n=5), or gAd (1.3 μg/mL; n=5). Values are the mean ± SEM.

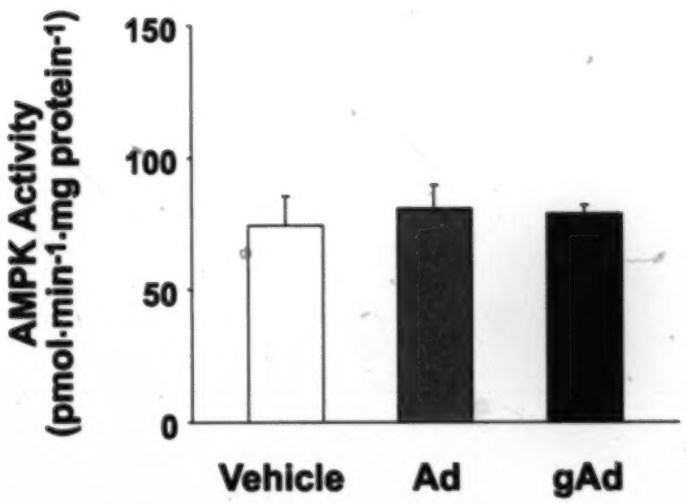
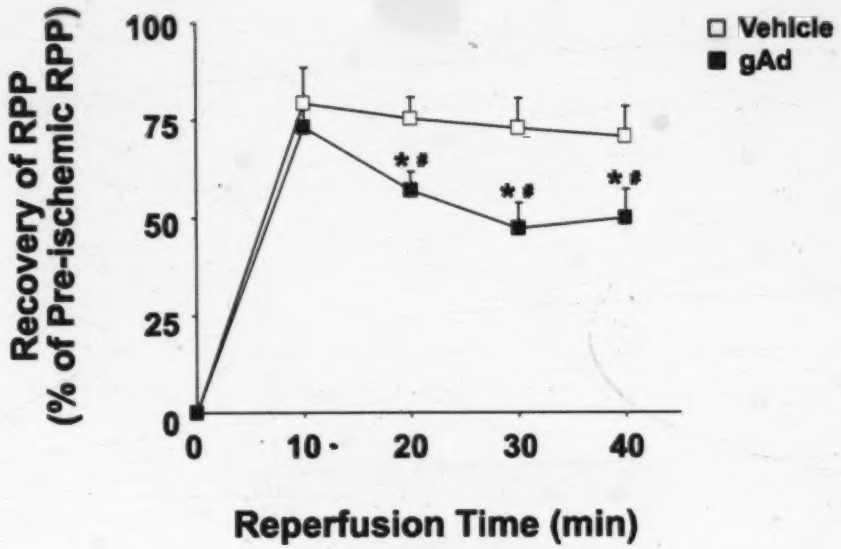

AMPK Activity
(pmol·min-1·mg protein-1)
150
100
50
0
Vehicle
Ad
gAd

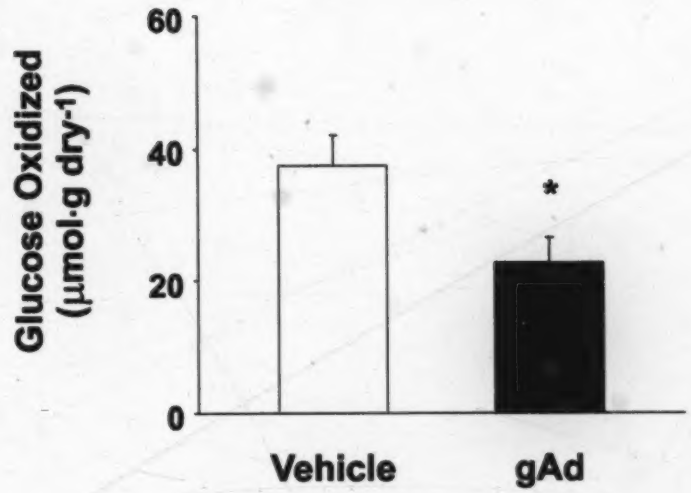
Figure 4.9 gAd Depresses the Contractile Recovery of Reperfused-Ischemic Hearts by Inhibiting Glucose Oxidation

Isolated working mouse hearts were subjected to a 30 min aerobic perfusion followed by 18 min of no-flow ischemia and 40 min of aerobic reperfusion. Vehicle (n=15) or gAd (n=13) was present during the entire perfusion. A) The recovery of the rate pressure product (RPP) during reperfusion is shown. Values are expressed as a percentage of pre-ischemic RPP. Values are the mean ± SEM. # denotes P<0.05 versus the 10 min time point of the same treatment group. * denotes P<0.05 versus vehicle at the same time point. B) The total amount of glucose oxidized during the 40 min of reperfusion in hearts perfused with vehicle (n=11) or gAd (n=7) is shown. Values are the mean ± SEM. * denotes P<0.05 versus vehicle-treated hearts.

A.


Recovery of RPP
(% of Pre-ischemic RPP)
100
75
50
25
0
0
10
20
30
40
Reperfusion Time (min)
Vehicle
gAd
*#
*#
*#

B.


Glucose Oxidized
(μmol·g dry-1)
60
40
20
0
*
Vehicle
gAd

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Chapter 5

Partial Purification of Novel Heart AMPKKs

5.1 Introduction

5'AMP-activated protein kinase (AMPK) plays an important role in the regulation of metabolism, protein synthesis and transcription [1]. AMPK has long been regarded as a cellular "energy sensor", since it is allosterically activated by increases in the intracellular AMP:ATP ratio, and thus is stimulated by ATP-depleting stresses [2]. AMPK is also regulated by extracellular cues, which do not alter the intracellular AMP:ATP ratio, but instead modulate AMPK phosphorylation at Thr 172 [2]. However, the molecular basis of phosphorylation-dependent regulation of AMPK activity remains to be elucidated.

AMPK is a heterotrimeric complex comprised of a catalytic subunit (α) and two regulatory subunits (β and γ) [3-5]. Multiple isoforms of each subunit have been cloned and the various isoforms differ in both their tissue and subcellular distribution profiles [6-10]. Thus far, eight phosphorylation sites have been identified among the heterotrimeric subunits. Five of these sites are situated on the β subunit. Specifically, β_1 is phosphorylated on Ser 24/25, Ser 96, Ser 101, Ser 108 and Ser 182 [11, 12]. With the exception of Ser 182, all of these residues are autophosphorylated by AMPK [11, 12]. Ser 182, which is neighbored by a proline residue, is not autophosphorylated but is proposed to be targeted by a Ser/Pro-directed protein kinase [11]. While the nonconservative

mutations S24A/S25A and S182A do not affect AMPK activity, these mutations promote the localization of the β_1 subunit to the nucleus [13]. Conversely, the S108A mutation leads to a decreased AMPK activity but does not affect the localization of the β_1 subunit [13]. Together, these data suggest that autophosphorylation of the β_1 subunit enhances AMPK activity and promotes a relocation of AMPK to the extranuclear sites. Moreover, phosphorylation of β_1 Ser 182 by an unidentified protein kinase may also direct the localization of AMPK.

The remaining three phosphorylation sites reside within the α catalytic subunit and have been identified as Thr 172, Thr 258, and Ser 485/Ser 491 (α_1/α_2) [12, 14]. All three sites are phosphorylated by purified liver AMPKK, but phosphorylation of Thr 172 is sufficient for activating AMPK [12]. This was established by mutagenesis experiments, which demonstrated that AMPKK stimulates α_1 T258E or S485E heterotrimers, but not α_1 T172E heterotrimers [12]. Moreover, the activities of T258E and S485E α_1 complexes were similar to wild-type complexes, whereas T172E α_1 complexes were substantially more active [12]. At present it is unclear whether each site is phosphorylated by one or more AMPKKs, as the purified liver AMPKK utilized in the aforementioned studies has been found to contain two AMPKKs ($_L$AMPKK1 and $_L$AMPKK2) [15].

Recent studies have established that the tumor suppressor protein kinase LKB1, also functions as an AMPKK [15-18]. LKB1 co-purifies with ʟAMPKK1 and ʟAMPKK2 [15]. *In vitro*, LKB1 phosphorylates Thr 172 of AMPK and thereby stimulates AMPK activity [16]. Furthermore, in LKB-deficient cells AMPK activity and Thr 172 phosphorylation are not stimulated by the AMPK-activating agents, phenformin, hydrogen peroxide, or 5-aminoimidazole-4-carboxamide riboside (AICAR) [15, 17, 18]. Thus, these data provide supporting evidence for the role of LKB1 as an AMPKK.

Notably however, other findings raise questions regarding the physiological role of LKB1 in regulating AMPK. Although the activities of muscle AMPKK and AMPK are stimulated by contraction [19], the activity of LKB1 is not affected [20]. Similarly, as demonstrated in Chapter 2, ischemia stimulates heart AMPKK and AMPK, but does not affect the activity of LKB1. In COS-7 cells, treatment with AMPK-activating agents does not cause LKB1 to be co-stimulated with AMPK [17]. Furthermore, in murine embryonic fibroblasts (MEFs) derived from LKB1 knockout mice, the basal activities of several AMPK-related kinases are dramatically reduced, whereas the activity of AMPK is similar between wild-type and LKB1-deficient MEFs [21]. Additionally, genetic and biochemical evidence demonstrate that Snf1, the *S. cerevisiae* orthologue of mammalian α AMPK, is phosphorylated and activated by three similar protein

kinases, Elm1, Pak1 and Tos3 [16, 22, 23]. Importantly, each of these protein kinases exhibit homology with mammalian protein kinases other than LKB1 [16].

Together, these data suggest that in addition to LKB1, other AMPKKs are key mediators of AMPK signaling in response to ischemia in the heart, and contraction in muscle. Thus, in this study we aimed to purify the non-LKB1 AMPKK(s) to facilitate the molecular characterization of these important AMPK-regulating kinases.

5.2 Materials and Methods

5.2.1 *AMPKK Assay*

The activity of AMPKK was determined by measuring the activation of the kinase domain of the α catalytic subunit (α_{312}), as described in Chapter 2.

AMPKK activity was also directly determined by measuring the phosphorylation of Thr 172 within α_{312}, as described in Chapter 2.

5.2.2 *PEG Fractionation of AMPKK*

To assess the distribution of AMPKK activity following PEG fractionation, three frozen powdered male Sprague Dawley rat hearts were homogenized in 6 volumes of homogenization buffer, which contained 50 mM Tris·HCl (pH 8 at 4°C), 10% (w/v) glycerol, 50 mM sodium fluoride, 5 mM sodium pyrophosphate,

10 mM β-glycerophosphate, 1 mM EDTA, 0.02% Brij-35, 1 mM DTT, and a protease inhibitor cocktail (Sigma). Heart tissue was homogenized twice for 30 seconds with a Polytron homogenizer. The homogenate was cleared by centrifugation at 13000 g for 30 min and the resulting supernatant was adjusted to contain a 6 mg/mL protein concentration by diluting the supernatant with homogenization buffer. The cleared homogenate was then adjusted to contain either 0, 5, 10 or 15% PEG6000 (w/v), was subsequently mixed for 1 h and centrifuged at 10000 g for 30 min. The supernatants were removed and the pellets were resuspended in homogenization buffer. Protein concentrations of the cleared homogenate, PEG6000 supernatants and PEG6000 precipitates were determined by the method of Bradford [24]. The activity of AMPKK was assessed in the cleared homogenate, PEG6000 supernatants and PEG6000 pellets.

5.2.3 *Immunoblotting*

Immunoblotting was conducted as described in Chapter 2, except the following primary antibodies, obtained from Cell Signaling Technologies, were utilized: anti-AMPK, anti-phosphoThr 172 AMPK, or anti-LKB1.

5.2.4 *Purification of AMPKK*

All procedures were conducted at 4°C unless otherwise indicated. The following buffers were used: buffer A contained 50 mM Tris·HCl (pH 8 at 4°C), 10% (w/v) glycerol, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 10 mM β-glycerophosphate, 1 mM EDTA, 0.02% Brij-35, 1 mM dithiothreitol; buffer B contained 50 mM Tris·HCl (pH 7.8 at 4°C), 10% (w/v) glycerol, 0.02% Brij-35, 1 mM dithiothreitol; buffer C was composed of 50 mM Tris·HCl (pH 7.6 at 4°C), 10% (w/v) glycerol, 1 mM EDTA, 0.02% Brij-35, 1 mM dithiothreitol.

Hearts were isolated from male Sprague Dawley rats, frozen in liquid nitrogen, and ground to a fine powder with a mortar and pestle. Approximately 200 g of frozen powdered heart tissue was homogenized in 1.2 L of buffer A containing a cocktail of protease inhibitors (Sigma). Hearts were homogenized twice for 30 s with a Polytron homogenizer and the homogenate was rehomogenized using a Potter-Elvehjem homogenizer. The homogenate was cleared by centrifugation at 13000 g for 30 min and the resulting supernatant was adjusted to contain a 6 mg/mL protein concentration by diluting the supernatant with buffer A. The cleared homogenate was then adjusted to contain 5% (w/v) PEG6000, stirred for 1 hour, and then centrifuged at 10000 g for 30 min.

All chromatographic separations were conducted using a Biologic HR chromatography system (Bio-Rad). At the end of a run, a protease inhibitor

cocktail (Sigma) was added to each collected fraction. One half of the 5% PEG 6000 supernatant was applied directly to a 400 mL DEAE sepharose FF (Amersham Biosciences) column, which was equilibrated in buffer A. Proteins were eluted with a 1.6 L linear gradient (0 to 400 mM) of NaCl in buffer A. Two peaks of AMPK kinase activity (termed hAMPKK1 and hAMPKK2) were resolved (see section 5.3). hAMPKK1 eluted with 90 mM NaCl while hAMPKK2 eluted with 190 mM NaCl. The same procedure was repeated for the second half of the 5% PEG supernatant. Fractions containing either hAMPKK1 or hAMPKK2 from both runs were pooled and dialyzed against buffer A. The subsequent chromatographic steps used to purify hAMPKK1 or hAMPKK2 were conducted in parallel.

Either hAMPKK1 or hAMPKK2 was loaded onto a 50 mL blue sepharose FF column which was equilibrated in buffer A. The column was washed with 150 mL of buffer A and proteins were eluted with a 500 mL linear gradient (0 to 1M) of NaCl in buffer A. Fractions containing AMPKK activity were pooled and dialyzed against buffer A and were then loaded onto a 25 mL Red Sepharose CL-6B (Amersham Biosciences). The column was washed with 100 mL of buffer A and proteins were eluted with a 150 mL linear gradient (0 to 1.8M) followed by a 25 mL linear gradient (1.8M to 3M) of NaCl in buffer A. Activity-containing fractions were pooled, dialyzed against buffer B, and then loaded onto a 10 mL

Source 30Q column (Amersham Biosciences). The Source 30Q column was washed with 150 mL of buffer B. A 100 mL linear gradient (0 to 250 mM) of NaCl followed by a 20 mL linear gradient (250 to 500 mM) of NaCl was applied to the column. Fractions containing AMPKK activity were pooled and dialyzed against buffer B containing 100 mM NaCl. Following dialysis, $CaCl_2$ was added to the sample to a final concentration of 2 mM. The sample was applied to a 10 mL Calmodulin Sepharose (Amersham Biosciences) column which was equilibrated with buffer B containing 100 mM NaCl and 2 mM $CaCl_2$. The column was washed with 40 mL of the equilibration buffer and bound proteins were eluted with 30 mL of buffer B containing 2 mM EGTA. The flow-through was pooled and diluted with 3 volumes of buffer C. One-third of the sample was then loaded onto a UnoQ1 column (Bio-Rad) equilibrated in buffer C containing 50 mM NaCl. The column was washed with 6 mL of equilibration buffer and then resolved with a 26 mL linear gradient (50 to 200 mM) of NaCl in buffer C. Aliquots of the fractions containing AMPKK activity were stored at -80°C.

5.2.5 *Autophosphorylation*

Equal volumes of hAMPKK1 fractions obtained from the UnoQ1 step were incubated in kinase buffer A for 0 or 60 min at 30°C. The reaction was terminated by the addition of SDS-PAGE loading buffer. Reactions were

subsequently boiled, and separated by SDS-PAGE. The gel was stained for phosphoproteins with ProQ Diamond (Molecular Probes) gel stain and visualized on a Molecular Imager FX (BioRad) imaging system.

5.2.6 *Two-Dimensional Polyacrylamide Gel Electrophoresis (2D PAGE)*

Equal volumes of hAMPKK1 fractions obtained from the UnoQ1 step were applied to immobilized pH gradient (IPG) strips (pH 3 to 10 linear gradient, BioRad), which were rehydrated and subjected to rapid voltage ramping, as described previously [25]. Following isoelectric focusing, IPG strips were equilibrated according to the manufacturer's instructions and embedded into pre-cast 4-15% gradient gels (BioRad) for electrophoresis. Resolved 2D gels were fixed and silver-stained using the Silver Stain Plus Kit (BioRad). All gels were stained together, to minimize staining variations. Stained gels were digitized with a calibrated densitometer (BioRad). Protein spots were detected, matched and quantitated with PDQuest 2-D Analysis Software (BioRad).

Protein spots with densities that correlated with the activity of hAMPKK1 were excised from the 2D gels and were submitted to the Institute for Biomolecular Design (University of Alberta) for trypsin digestion and LC/MS/MS mass spectrometric analyses. Mascot generic format files of completed

LC/MS/MS runs were used with the MASCOT software package (Matrix Science) for database searches and protein identification.

5.3 Results and Discussion

5.3.1 *PEG Fractionation of AMPKK*

To ascertain an appropriate starting point for the purification of LKB1-distinct AMPKKs, we determined whether AMPK and LKB1, but not other AMPKKs, could be selectively precipitated with PEG6000. Heart homogenates were precipitated using 0, 5, 10, or 15% PEG6000 and the resulting supernatants and precipitates were assayed for AMPKK activity and immunoblotted for AMPK and LKB1. Although the greatest amount of AMPKK activity was detected in the homogenate itself (Figure 5.1A), a significant amount of AMPK and LKB1 were also detected (Figures 5.1B and 5.1C, respectively). When the amount of PEG6000 was increased, an increasing amount of AMPKK activity, AMPK and LKB1 were found in the precipitates (Figure 5.1A, 5.1B, and 5.1C). However, while the majority of AMPK and LKB1 are precipitated with 5% PEG6000 (Figure 5.1B and 5.1C), 60% of the total AMPKK activity still resided in the supernatant (Figure 5.1 A). Thus, the 5% PEG6000 supernatant is an attractive source for the purification of LKB1-distinct AMPKK, as it lacks endogenous AMPK and LKB1.

5.3.2 *Purification of AMPKK*

In contrast to Hawley *et al.* [14], following anion-exchange chromatography with DEAE, two peaks of AMPKK activity were resolved (Figure 5.2). The peaks were named hAMPKK1 and hAMPKK2 based on their order of elution. Specifically, hAMPKK1 eluted with 90 mM NaCl, whereas hAMPKK2 eluted with 190 mM NaCl. hAMPKK1 and hAMPKK2 continued to exhibit different chromatographic behaviour on both blue (Figure 5.3A and 5.3B, respectively) and red sepharose columns (Figure 5.3C and 5.3D, respectively). hAMPKK1 eluted from the blue sepharose column with 930 mM NaCl while hAMPKK2 eluted with 190 mM NaCl. On the red sepharose column, hAMPKK1 also bound more strongly than hAMPKK2.

In contrast to the first anion-exchange step, hAMPKK1 and hAMPKK2 exhibited similar elution profiles following separation on the Source 30Q column (Figure 5.4A and 5.4B). Indeed multiple peaks of activity were observed with hAMPKK2 (Figure 5.4B), however the greatest peak of activity eluted with 85 mM NaCl which is similar to the 90 mM NaCl required to elute hAMPKK1 (Figure 5.4B and 5.4A). The similar elution conditions required may be attributed to hAMPKK1 and hAMPKK2 having more similar charges at pH 7.8 than at 8.0. Separation on calmodulin sepharose primarily functioned as negative chromatography (Figure 5.4C and 5.4D); however, a small peak of

activity was also retained on the column (Figure 5.4C and 5.4D). This peak of activity may correspond to Ca^{2+}/calmodulin-dependent protein kinase kinase, which has been shown to activate AMPK [26]. Like the Source 30Q column, chromatography on the UnoQ1 column resolved multiple peaks of activity for HAMPKK2, but the greatest activity eluted with identical NaCl concentrations required for eluting HAMPKK1 (Figure 5.5A and 5.5B).

In addition to exhibiting different chromatographic behaviour, HAMPKK1 and HAMPKK2 differed in activity. Throughout the purification, HAMPKK2 was less active than HAMPKK1. A possible explanation for HAMPKK2 exhibiting less activity is that HAMPKK2 may have a higher K_m for α_{312} than HAMPKK1.

5.3.3 *LKB1 does not Co-Purify with Heart AMPKK*

Since LKB1 is an AMPKK, we determined whether the residual LKB1 present in the 5% PEG supernatant co-purifies with HAMPKK1 or HAMPKK2. AMPKK-containing fractions obtained throughout the purification procedure were immunoblotted for LKB1 (Figure 5.6). Consistent with our findings discussed above, the 5% PEG supernatant did not contained less amounts of LKB1 than the homogenate. During the early stages of purification, LKB1 did co-purify with HAMPKK1 and was enriched following purification on the blue sepharose column (Figure 5.6). However, the amount of detected LKB1 protein

did not correlate with the fold purification of HAMPKK1. Moreover, LKB1 was separated from HAMPKK1 at the red and Source 30Q step and was not detected after the UnoQ1 stage (Figure 5.6). In contrast, LKB1 co-purified with HAMPKK2 through the first column and was not detected with subsequent fractionation. Importantly, these data demonstrate that LKB1 is not the only AMPKK present in the heart.

Previously, it was shown that LKB1 co-purifies with liver AMPKK. It was demonstrated that LKB1 co-fractionates with both LAMPKK1 and LAMPKK2 after a third chromatographic step. The discrepancy between our findings and previous findings may be due to differences in the purity of the preparations used. In the previous study, LAMPKK1 and LAMPKK2 were not resolved until the third column [15], while in the present study two AMPKKs were resolved on the first column. Moreover, the previous study did not examine whether LKB1 co-purifies with LAMPKK1 and LAMPKK2 with further purification [15], whereas we find that LKB1 co-purifies with AMPKK during the early stages, but not the later stages of purification. Alternatively, LKB1 may be the only AMPKK present in the liver.

5.3.4 *Elucidation of Candidate AMPKK Proteins*

Since hAMPKK1 was more active and was purified to a greater degree than hAMPKK2, we focused further investigations on hAMPKK1. To elucidate candidate hAMPKK1 proteins, fractions containing hAMPKK1 were allowed to autophosphorylate for 0 or 60 min (Figure 5.7B). As shown in Figure 5.7B, the abundance of an autophosphorylating protein with an approximate molecular weight of 50 kDa correlated with the activity of hAMPKK1 (Figure 5.7A).

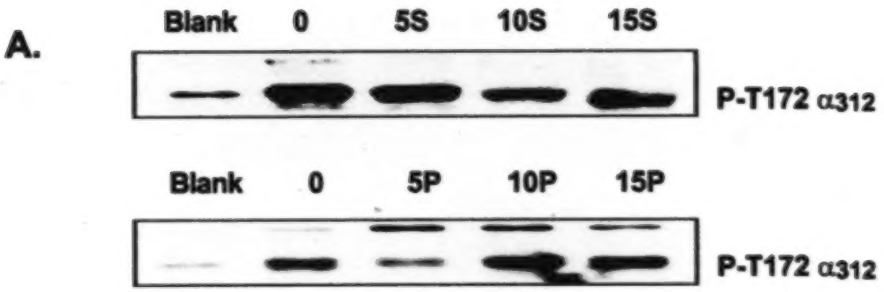
hAMPKK1 fractions were also separated by 2D gel electrophoresis to identify proteins that fractionate with AMPKK activity. Analysis of the 2D gels identified a candidate protein spot that elutes in proportion with the activity of AMPKK (Figure 5.7C). Bioinformatic analysis of the mass spectrometry data obtained from this protein revealed that it is not a known protein.

5.3.5 *Summary*

In this study, we demonstrate that two AMPKKs (hAMPKK1 and hAMPKK2) can be purified from heart. In addition, we show that LKB1 does not co-purify with either hAMPKK1 or hAMPKK2. We further demonstrate that hAMPKK1 is an unknown protein. The data presented herein will enable the molecular identification and characterization of these novel AMPKKs.

Figure 5.1 PEG6000 Fractionation of AMPKK Activity, LKB1 and AMPK

Rat heart homogenate was precipitated with 0, 5, 10, or 15% PEG6000. A) AMPKK activity in PEG6000 supernatants and precipitates is shown. AMPKK activity was measured by detecting an increase in the phosphorylation of Thr172 of α_{312}. The basal Thr 172 phosphorylation state of α_{312} is also shown (Blank). B) Distribution of AMPKK activity (relative to total activity) following PEG6000 precipitation is depicted. C) Immunoblots for LKB1 and AMPK in PEG6000 supernatants and precipitates are shown. 0 denotes homogenate. 5S, 10S, and 15S denote 5, 10, and 15% PEG6000 supernatants, respectively. 5P, 10P, and 15P denote 5, 10, and 15% PEG6000 precipitates, respectively.


A.
Blank
0
5S
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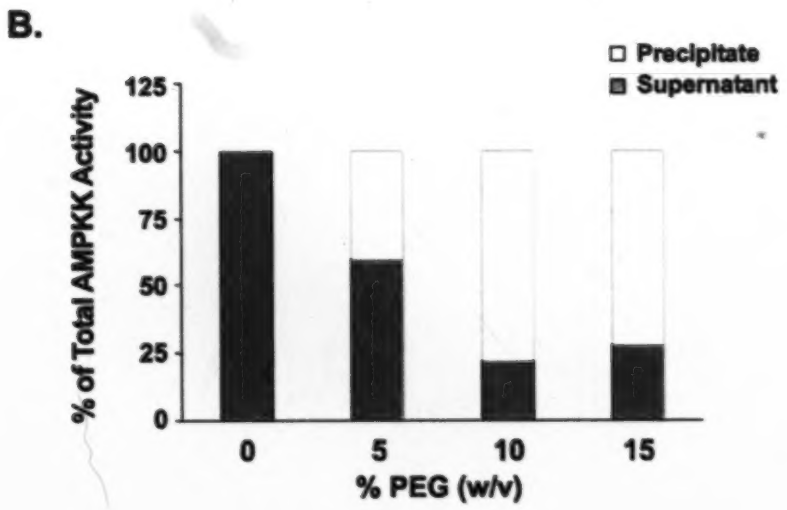

B.
Precipitate
Supernatant
% of Total AMPKK Activity
125
100
75
50
25
0
0
5
10
15
% PEG (w/v)

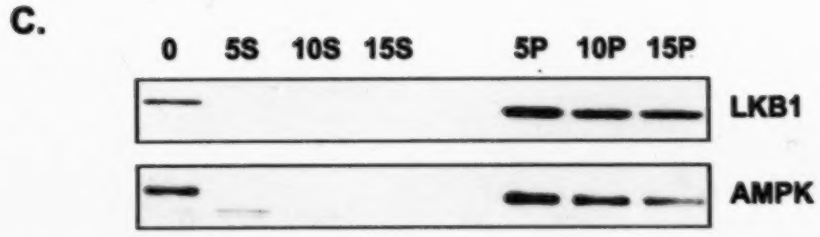

C.
0
5S
10S
15S
5P
10P
15P
LKB1
AMPK

Figure 5.2 Two Peaks of AMPKK Activity are Resolved on DEAE Sepharose

Chromatogram depicting UV_{280}, conductivity and AMPKK activity following fractionation of 5% PEG6000 supernatant on DEAE sepharose is shown.

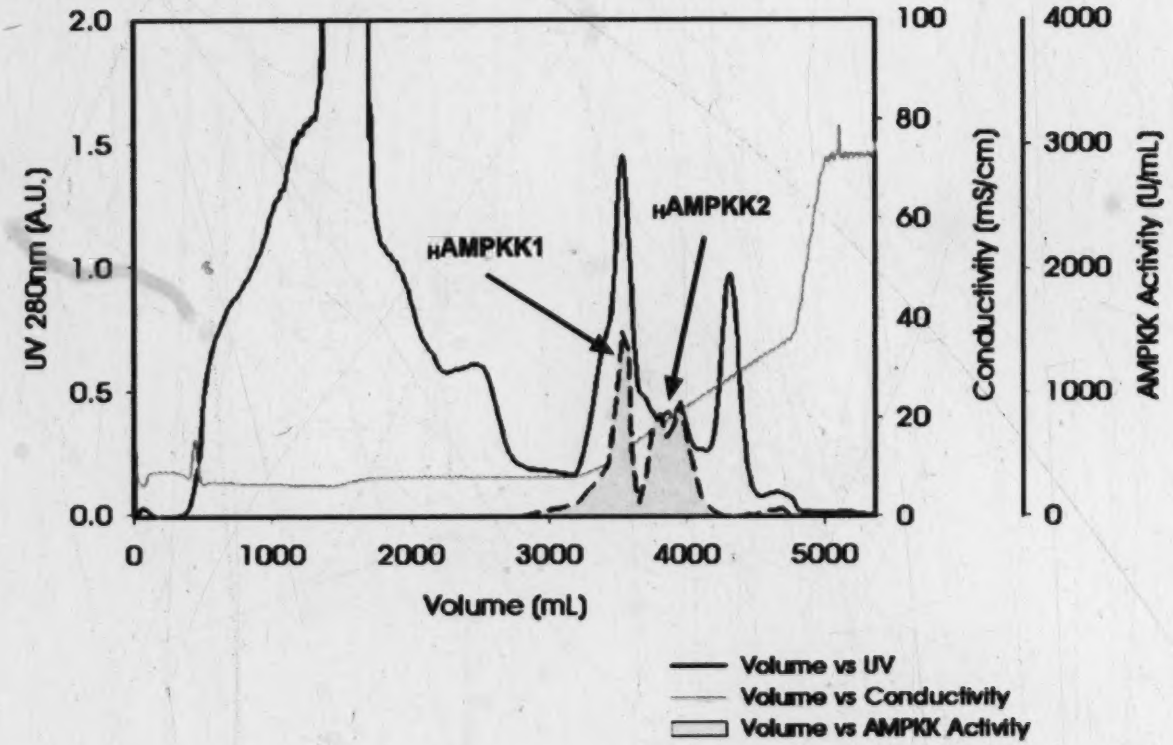
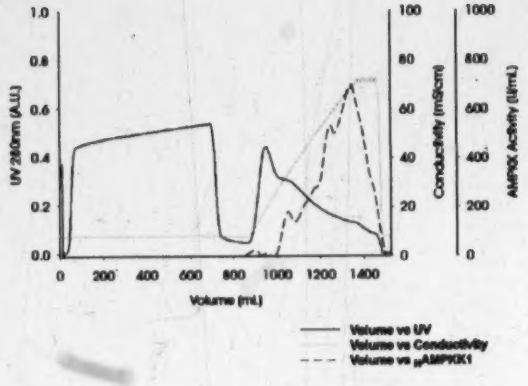

2.0
1.5
1.0
0.5
0.0
UV 280nm (A.U.)
100
80
60
40
20
0
Conductivity (mS/cm)
4000
3000
2000
1000
0
AMPKK Activity (U/mL)
0
1000
2000
3000
4000
5000
Volume (mL)
hAMPKK1
hAMPKK2
Volume vs UV
Volume vs Conductivity
Volume vs AMPKK Activity

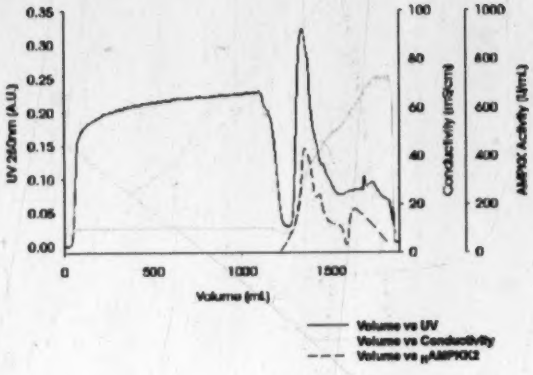
Figure 5.3 hAMPKK1 and hAMPKK2 Differentially Bind to Blue and Red Sepharose

Chromatograms depicting UV_{280}, conductivity and hAMPKK1 activity following fractionation on blue (A) and red sepharose (C) are shown. Chromatograms depicting UV_{280}, conductivity and hAMPKK2 activity following fractionation on blue (B) and red sepharose (D) are shown.

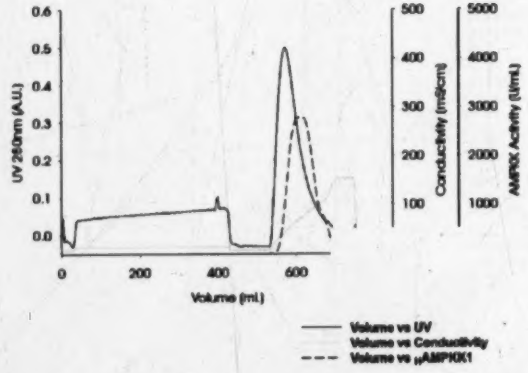
A.


UV 280nm (A.U.)
Conductivity (mS/cm)
AMPKX Activity (U/mL)
Volume (mL)
Volume vs UV
Volume vs Conductivity
Volume vs μAMPKX1

B.


UV 280nm (A.U.)
Conductivity (mS/cm)
AMPKX Activity (U/mL)
Volume (mL)
Volume vs UV
Volume vs Conductivity
Volume vs μAMPKX2

C.


UV 280nm (A.U.)
Conductivity (mS/cm)
AMPKX Activity (U/mL)
Volume (mL)
Volume vs UV
Volume vs Conductivity
Volume vs μAMPKX1

D.

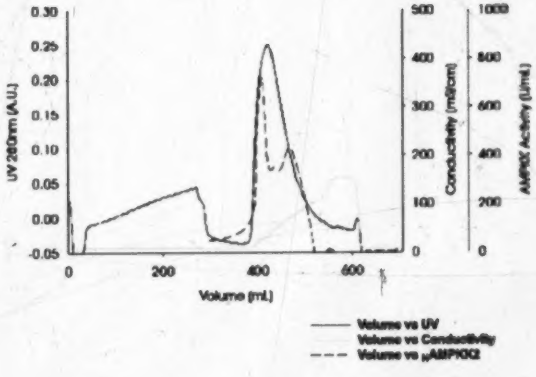
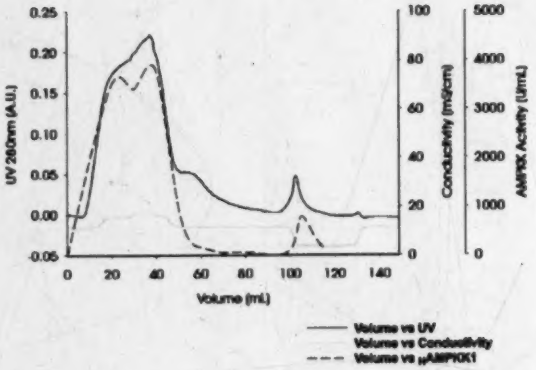

UV 280nm (A.U.)
Conductivity (mS/cm)
AMPKX Activity (U/mL)
Volume (mL)
Volume vs UV
Volume vs Conductivity
Volume vs μAMPKX2

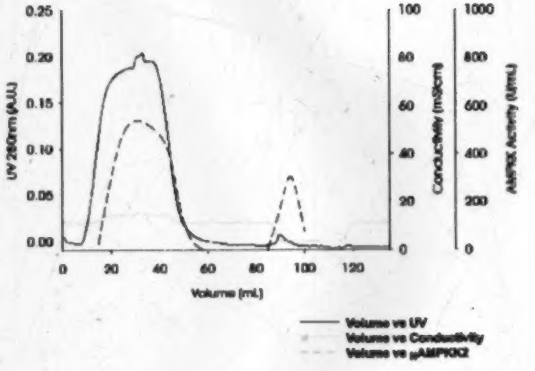
Figure 5.4 $_{H}$AMPKK1 and $_{H}$AMPKK2 Purification on Calmodulin Sepharose and Source 30Q Columns

Chromatograms depicting UV_{280}, conductivity and $_{H}$AMPKK1 activity following fractionation on calmodulin sepharose (A) and source 30Q (C) are shown. Chromatograms depicting UV_{280}, conductivity and $_{H}$AMPKK2 activity following fractionation on calmodulin sepharose (B) and source 30Q (D) are shown.

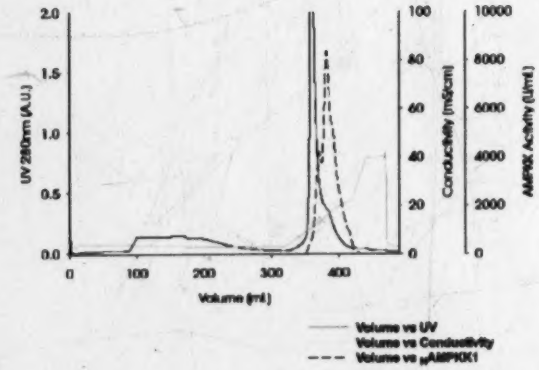
A.


UV 280nm (A.U.)
Conductivity (mS/cm)
AMPKK Activity (U/mL)
Volume (mL)
Volume vs UV
Volume vs Conductivity
Volume vs μAMPKK1

B.


UV 280nm (A.U.)
Conductivity (mS/cm)
AMPKK Activity (U/mL)
Volume (mL)
Volume vs UV
Volume vs Conductivity
Volume vs μAMPKK2

C.


UV 280nm (A.U.)
Conductivity (mS/cm)
AMPKK Activity (U/mL)
Volume (mL)
Volume vs UV
Volume vs Conductivity
Volume vs μAMPKK1

D.

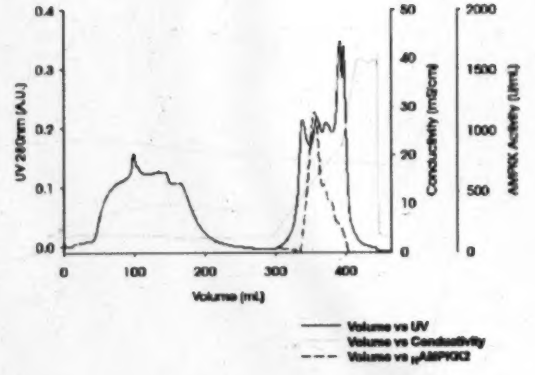
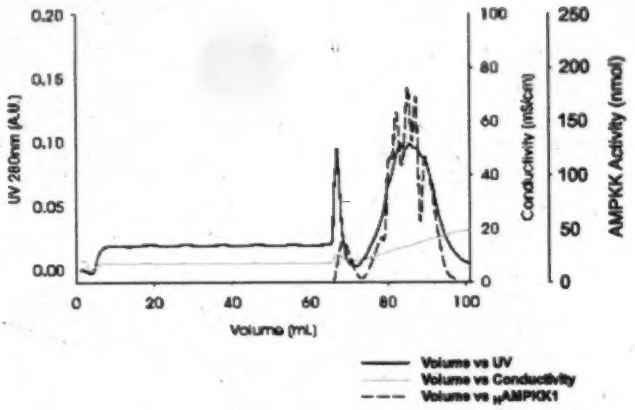

UV 280nm (A.U.)
Conductivity (mS/cm)
AMPKK Activity (U/mL)
Volume (mL)
Volume vs UV
Volume vs Conductivity
Volume vs μAMPKK2

Figure 5.5 hAMPKK1 and hAMPKK2 Purification on UnoQ1

Chromatograms depicting UV_{280}, conductivity and hAMPKK1 (A) or hAMPKK2 activity (B) following fractionation on a UnoQ1 column are shown (A and B, respectively).

A.


0.20
0.15
0.10
0.05
0.00
UV 280nm (A.U.)
100
80
60
40
20
0
Conductivity (mS/cm)
250
200
150
100
50
0
AMPKK Activity (nmol)
0
20
40
60
80
100
Volume (mL)
Volume vs UV
Volume vs Conductivity
Volume vs μAMPKK1

B.

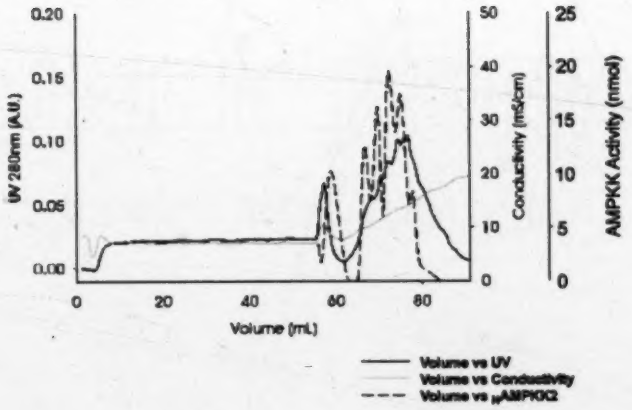
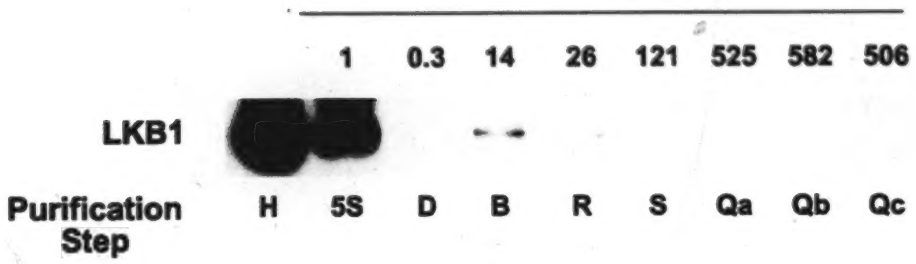

0.20
0.15
0.10
0.05
0.00
UV 280nm (A.U.)
50
40
30
20
10
0
Conductivity (mS/cm)
25
20
15
10
5
0
AMPKK Activity (nmol)
0
20
40
60
80
Volume (mL)
Volume vs UV
Volume vs Conductivity
Volume vs μAMPKK2

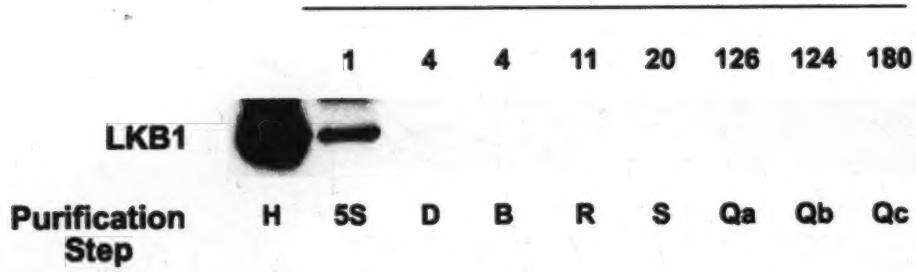
Figure 5.6 LKB1 does not Co-Purify with $_{H}$AMPKK1 or $_{H}$AMPKK2

LKB1 immunoblot of $_{H}$AMPKK1- and $_{H}$AMPKK2-containing fractions obtained throughout the purification: homogenate (5μg), 5% PEG6000 supernatant (5 μg), and chromatography on DEAE sepharose (1 μg), blue sepharose (1μg), red sepharose (1 μg), source 30Q (1 μg), and UnoQ1 (1 μg).

Fold Purification of $_H$AMPKK1



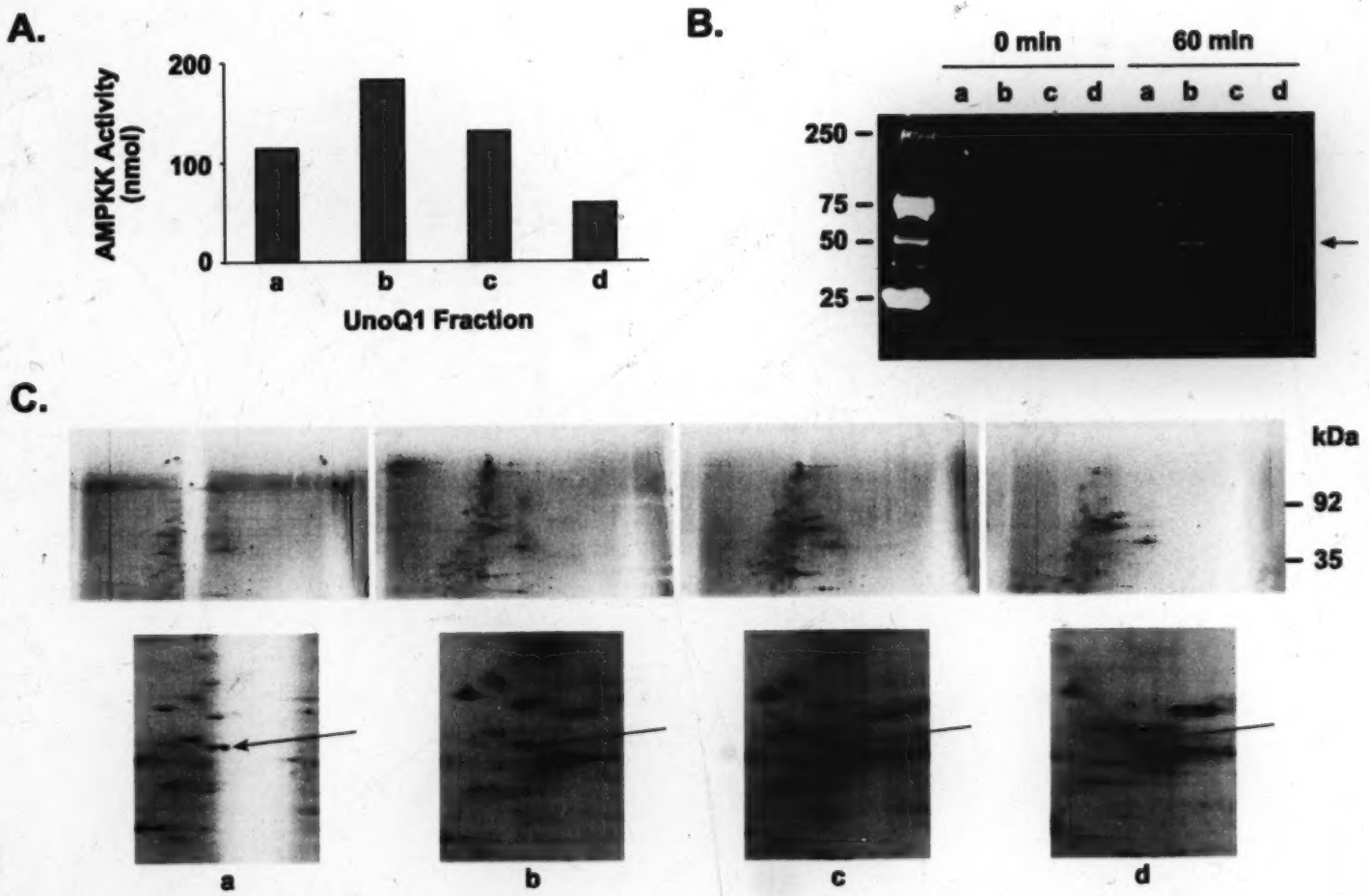
Fold Purification of $_H$AMPKK2



H=homogenate
5S=5% PEG6000 supernatant
D=DEAE Sepharose
B=Blue Sepharose
R=Red Sepharose
S=Source 30Q
Qa=UnoQ1 fraction a
Qb=UnoQ1 fraction b
Qc=UnoQ1 fraction c

Figure 5.7 Elucidation of Candidate hAMPKK1 Proteins

A) hAMPKK1 activity of fractions obtained following chromatography on a UnoQ1 column is shown. B) Autophosphorylation of hAMPKK1 UnoQ1 fractions after 0 min and 60 min are shown. A candidate hAMPKK1 protein is indicated by an arrow. C) Silver-stained 2D gels (upper) of hAMPKK1 UnoQ1 fractions. Lower panels represent a zoomed in region of the 2D gel. A candidate hAMPKK1 protein is indicated by an arrow.


A.
AMPKK Activity (nmol)
200
100
0
a
b
c
d
UnoQ1 Fraction
B.
0 min
60 min
a b c d a b c d
250
75
50
25
C.
kDa
92
35
a
b
c
d

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Chapter 6

DISCUSSION

6.1 Summary

5'AMP-activated protein kinase (AMPK) is an evolutionarily conserved Ser/Thr protein kinase [1] that coordinates glucose and fatty acid metabolism in the mammalian heart [2]. Importantly, previous work established a pivotal role for AMPK in stimulating the metabolic sequelae, which ensue during and following ischemia in the heart [3-6]. Prior studies also demonstrated that insulin signaling through Akt , negatively regulates AMPK in the heart [7, 8]. Other studies linked the hormone adiponectin [9] and the globular domain of adiponectin (gAd) to AMPK signaling in skeletal muscle [9], which provided evidence that AMPK activation is also mediated by receptor signaling. Despite these significant advancements in our understanding of AMPK, the molecular mechanisms that regulate AMPK activity in the heart have not previously been clearly elucidated. Hence the overall objective of this thesis was to further characterize and define the mechanisms that regulate myocardial AMPK activity.

During myocardial ischemia, activation of 5'AMP-activated protein kinase (AMPK) leads to the stimulation of glucose uptake [5, 10], glycolysis [5, 6] and fatty acid oxidation [3, 4]. Although AMPK signaling in the ischemic heart is well characterized [3-6, 10], the relative contribution of positive allosterism by the ratios of AMP:ATP and Cr:PCr, and activating phosphorylation by AMPK kinase (AMPKK), to the regulation of AMPK during ischemia is unknown. Thus,

in Chapter 2 we examined the effects of severe and mild ischemia on AMPK regulation in isolated working rat hearts. In hearts subjected to severe ischemia, the ratios of AMP:ATP and Cr:PCr were significantly elevated as compared to aerobic hearts. However, the ratios of AMP:ATP and Cr:PCr were unaltered by mild ischemia. Severe ischemia stimulated AMPK signaling, as demonstrated by an increase in both AMPK activity and acetyl-CoA carboxylase (ACC) phosphorylation. Importantly, both AMPKK activity and AMPK phosphorylation were increased by severe ischemia. Although mild ischemia did not alter the ratios of AMP:ATP and Cr:PCr, mild ischemia stimulated AMPK activity, AMPK phosphorylation and AMPKK activity. We also investigated whether the recently identified AMPKK, LKB1 is also stimulated by ischemia. Although LKB1 phosphorylated and activated a recombinant catalytic domain of AMPK, the protein abundance and activity of LKB1 was similar between aerobic and severe ischemic hearts. In summary, we demonstrate that myocardial ischemia stimulates AMPK via an AMPKK other than LKB1. Our data emphasize the critical role AMPKK plays in mediating AMPK signaling during myocardial ischemia.

In contrast to ischemia, insulin has been found to inhibit the activity and/or phosphorylation of AMPK in the heart [7, 8, 11]. The inhibition of AMPK by insulin has been shown to occur through a non-allosteric mechanism [8] and

is mimicked by stimulation of Akt [7]. Since insulin and Akt inhibit AMPK phosphorylation, we investigated whether insulin signaling decreases the activity of AMPKK and thereby inhibits AMPK phosphorylation. Thus, we determined whether insulin represses the activity of AMPKK in aerobically-perfused hearts. Surprisingly, in aerobic hearts, AMPK phosphorylation was similar in insulin-treated and control hearts. In agreement with the absence of an effect on AMPK, insulin also did not inhibit the activity of AMPKK or the phosphorylation of ACC in aerobic hearts. Since, it has been reported that insulin exerts a more pronounced inhibition of AMPK phosphorylation in ischemic hearts than aerobic hearts [8], the effects of insulin on AMPKK activity and AMPK signaling were also determined in hearts subjected to mild ischemia. Similarly, in control and insulin-treated ischemic hearts, the activity of AMPKK, the phosphorylation of AMPK and the phosphorylation of ACC were similar. Importantly, fatty acids have recently been shown to antagonize the inhibition of AMPK by insulin [12]. Although we did not examine the regulation of AMPK by insulin in hearts perfused without fatty acids, our data provides further evidence that insulin does not inhibit AMPKK or AMPK signaling in aerobic or ischemic hearts perfused with clinically relevant levels of fatty acids.

For many years, AMPK was an orphan kinase as it was not established as an integral component of hormone-receptor signaling. Recently however, AMPK

was identified as a crucial effector of adiponectin- and/or gAd-receptor signaling in skeletal muscle [13]. Yet, it was unknown whether adiponectin and gAd exert similar regulatory effects on metabolism in the adult heart. Thus, we examined the signaling, metabolic and functional effects of adiponectin and gAd in the adult heart. In aerobically-perfused hearts, neither adiponectin nor gAd altered mechanical function. Rates of glycolysis were also similar among vehicle-, adiponectin- and gAd-treated hearts. Notably, adiponectin and gAd reciprocally regulated glucose oxidation. In adiponectin-treated hearts glucose oxidation was stimulated when compared to vehicle-treated hearts. Conversely, glucose oxidation rates were inhibited by gAd treatment. Consistent with the Randle cycle [14], palmitate oxidation rates were stimulated in gAd-treated hearts when compared with vehicle-treated hearts. Although adiponectin did not affect AMPK activity, adiponectin stimulated Akt phosphorylation in an insulin-dependent manner. Conversely, gAd inhibited Akt phosphorylation in an insulin-dependent manner. Moreover, in the absence of insulin no differences in glucose oxidation rates were evident among vehicle-, adiponectin-, or gAd-treated hearts.

Depressed rates of glucose oxidation and/or increased rates of palmitate oxidation have been shown to impair the recovery of mechanical function in repefused-ischemic hearts [15]. Thus, we hypothesized that gAd treatment

would impair the contractile recovery of ischemic-reperfused hearts. In accordance, gAd inhibited glucose oxidation during reperfusion and impaired the mechanical recovery of reperfused-ischemic hearts when compared with vehicle-treatment. In conclusion, these data demonstrate that adiponectin stimulates glucose oxidation in the heart by enhancing insulin signaling through Akt. Conversely, gAd inhibits glucose oxidation by repressing insulin-stimulated Akt phosphorylation and by stimulating palmitate oxidation.

In 1978, it was first demonstrated that phosphorylation of AMPK plays an important role in stimulating AMPK activity [16]. Recent advances have further established that AMPK is phosphorylated at Thr 172 by the upstream kinase, AMPKK [17]. Additionally, several groups have elucidated that the tumor suppressor kinase LKB1, functions as AMPKK [18-21]. Importantly, it has been shown that LKB1 is not co-regulated with AMPK in various cell types [19, 22]. Furthermore, in Chapter 2 we demonstrated that ischemia-induced activation of AMPK is not mediated by LKB1, but is effected through an alternate AMPKK. Thus, in Chapter 5 we partially purified LKB1-distinct AMPKKs from heart. Intriguingly, two AMPKKs were resolved by an initial fractionation by anion-exchange chromatography. The AMPKKs were named hAMPKK1 and hAMPKK2, based on their order of elution. Notably, throughout the purification hAMPKK1 was more active than hAMPKK2. In addition, hAMPKK1 and

hAMPKK2 displayed different chromatographic behaviours. Specifically, hAMPKK1 was retained longer on both blue and red sepharose columns than hAMPKK2. Immunoblot analysis of purified hAMPKK1 and hAMPKK2 obtained throughout the purification revealed that a small of amount of LKB1 co-purified with hAMPKK1 and hAMPKK2 during the early stages but not the later stages of purification. Since hAMPKK1 exhibited greater activity than hAMPKK2, we further characterized the identity of this protein. A candidate hAMPKK1 protein was identified by two-dimensional gel electorophoresis, and mass spectrometric analysis demonstrated that this protein is unknown and is not LKB1. In this study, we demonstrate that three AMPKKs, LKB1, hAMPKK1 and hAMPKK2, regulate AMPK in the heart. Furthermore, we establish that hAMPKK1 is an unknown protein. Importantly, this study will facilitate the molecular cloning of hAMPKK1 and hAMPKK2.

6.2 Study Limitations and Future Directions

Data in this thesis provide significant insight into the molecular regulation of cardiac AMPK activity. However, some limitations in the studies should be considered. First, the isolated working heart preparation was utilized to study AMPK signaling. Although the perfusion conditions employed mimic the plasma levels of fatty acids, glucose, and insulin in the rat or mouse, it is unclear

whether the findings presented occur *in vivo*. Since all of the studies were conducted with rodent hearts, it is also uncertain whether AMPK is regulated by similar mechanisms in the human heart. Importantly, in efforts to build upon the regulatory mechanisms presented herein, future studies should focus on three aspects of AMPK signaling regulation that were not examined in this thesis. Specifically, the temporal and spatial aspects of AMPK regulation, as well as the roles of distinct AMPK subunit isoforms should be investigated.

In Chapter 2, while it was evident that AMPK was phosphorylated and activated by mild ischemia, this did not translate to an increase in ACC phosphorylation. One possible explanation is that AMPK may have undergone transient activation and was inactive at the time of assay. Alternatively, mild ischemia may not stimulate the translocation of AMPK and/or ACC to the same subcellular region. Another possibility is that the specific AMPK heterotrimer stimulated by mild ischemia does not phosphorylate ACC. This is also plausible as the α_2 heterotrimer does not phosphorylate ACC *in vitro* [23].

In Chapter 3, we demonstrate that AMPKK activation during ischemia is not inhibited by insulin. Furthermore, we did not find any evidence that insulin regulates AMPKK or AMPK in aerobic hearts treated for 60 min. However, we did not investigate whether insulin inhibits AMPKK or AMPK in hearts treated for shorter times. Furthermore, since AMPK associates with glycogen and

insulin stimulates glycogen synthesis, insulin may inhibit AMPK signaling by sequestering AMPK to glycogen particles. Alternatively, insulin may inhibit the Thr 172 phosphorylation of α_1, but not α_2, or vice versa. Recently Clark *et al.* demonstrated that insulin inhibition of myocardial AMPK is abrogated by high levels of circulating fatty acids [12]. In our study, hearts were perfused with 1.2 mM palmitate to mimic the high levels of fatty acids seen in clinical myocardial ischemia [24-27]. Thus, it is possible that in our study the inhibition of AMPK by insulin was antagonized by fatty acids in the perfusate, and warrants further investigation.

In Chapter 4, we establish that adiponectin and the globular domain of adiponectin (gAd) recipirocally regulate glucose oxidation through an AMPK-independent signaling pathway. Specifically, neither adiponectin nor gAd altered AMPK activity in hearts treated for 40 min or for 10 min. However, adiponectin stimulated Akt Thr 308 phosphorylation and glucose oxidation in an insulin-dependent manner; thus, highlighting the importance of other signaling pathways in the regulation of myocardial glucose metabolism. Although the regulation of Akt is not central to the objective of this thesis, future investigations should delineate which receptors (AdipoR1, AdipoR2, or T-cadherin) enhance insulin signaling to Akt.

A major finding presented in this thesis is that LKB1 is not the sole mammalian AMPKK. We demonstrate that ischemia stimulates an AMPKK that is distinct from LKB1. Moreover, we establish that two additional AMPKKs, hAMPKK1 and hAMPKK2, can be purified from heart and we further show that LKB1 does not co-purify with hAMPKK1 or hAMPKK2. Since an LKB1-distinct AMPKK and/or AMPKKs mediate the activation of AMPK by ischemia, future studies should focus on the characterization and identification of these novel AMPKKs. Although only one candidate hAMPKK1 was identified by correlating the activity of hAMPKK1 with the abundance of individual proteins, we cannot exclude the possibility that the candidate protein is not hAMPKK1. In-gel kinase assays, autophosphorylation studies, far-western blotting experiments, and sequencing by N-terminal Edman degradation will aid in determining whether the candidate protein is hAMPKK1. Establishing the identity of alternate AMPKKs will provide further insight into the mechanisms by which ischemia signals to AMPK.

6.3 Conclusion

In conclusion, this thesis defines the mechanisms that regulate myocardial AMPK in response to ischemia, insulin, adiponectin, and gAd. Furthermore, we demonstrate the importance of AMPKK in mediating AMPK signaling in the

ischemic heart, and have elucidated that three AMPKKs exist in the heart. Importantly, these studies also illustrate the multifaceted regulation of myocardial metabolism by AMPK-independent pathways, and provide the impetus for future in-depth studies in this essential area of research.

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